

UNCLASSIFIED

AD NUMBER
AD833598
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies and their contractors; Critical Technology; 29 SEP 1966. Other requests shall be referred to Department of the Army, Fort Detrick, MD.
AUTHORITY
SMUFD, D/A ltr, 15 Feb 1972

THIS PAGE IS UNCLASSIFIED

AD83508

TRANSLATION NO.1799

~~XXXXXX~~ CA-18-064-D6-00030(A)

DATE: 29 September 1966

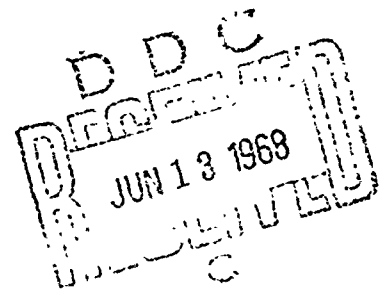
DDC AVAILABILITY NOTICE

Reproduction of this publication in whole or in part is prohibited. However, DDC is authorized to reproduce the publication for United States Government purposes.

STATEMENT #2 UNCLASSIFIED

This document is subject to special export controls and each transmittal to foreign governments or foreign nationals may be made only with prior approval of DEPARTMENT OF THE ARMY

Fort Detrick
Frederick, Maryland 21701



#1799

y.1

CA-18-064-D6-00030(A)

T-428-1

29 September 1966

FLUORESCENCE MICROSCOPY AND MICROBIOLOGY

This translation is protected by international copyright law and is provided solely for internal use at these laboratories. Reproduction or release by recipients is prohibited. Queries concerning availability should be directed to the Technical Releases Branch, Technical Information Division

FOR DESK USE ONLY. Not for use in Dept of Army publication. See AR 310-1, Par. 16.

**Best
Available
Copy**

FLUORESCENCE MICROSCOPY AND MICROBIOLOGY
Siegfried Strugger

M. & H. Schaper Publishing House
1949

Hannover

CONTENTS

FOREWORD	1
INTRODUCTION	2
PRINCIPLES OF FLUORESCENCE MICROSCOPY	3
Fluorescence	3
The Fluorescence Microscope	6
The Blue-Light Fluorescence Microscope	8
The UV-Light Fluorescence Microscope	13
Practical Use of the UV and Blue-Light Microscopes ...	15
FLUOROCHROMING OF FIXED BACTERIAL SMEAR PREPARATIONS	16
Introduction	16
Definitions and Characteristics of Fluorochromes	16
The Theory of Fluorochroming of Bacterial Smears	22
Model Tests	22
pH Dependence of Fluorochroming of Smear Preparations with Cathodic, Anodic, and Electroneutral Fluoro- chromes	27
Theory	31
The Practice of Fluorochroming Fixed Bacterial Smears ..	32
A General Method of Fluorochroming Bacterial Smears ..	32
Fluorochroming of Bacterial Spores	36
Elective Fluorochroming of <u>Mycobacterium tuberculosis</u> and Other Acid-Fast Types	38
Elective Fluorochroming of Trypanosomes in Blood Smears	43
Fluorochroming of Virus Particles	44
FLUOROCHROMING OF LIVING MICROORGANISMS (ACRIDINE ORANGE METHOD)	44
Introduction	44
Chemistry and Characteristics of Acridine Orange	48
Characteristics as a Diachrome	48
Characteristics as a Fluorochrome	50
Influence of the pH Value on Fluorescence	51
Lipid Solubility	54

Fluorochroming of Models with Acridine Orange	54
The Cellulose Model	54
The Gelatin-Toluene Model	56
Fundamental Experiment on the Upper Epidermis of <u>Allium cepa</u> Scale for Vital Fluorochroming of Cells with Acridine Orange	57
Analysis of the Concentration Effect in Living and Dead Cells	63
Evaluation of Methods	66
The Theory of Acridine Orange Fluorochroming of Protoplasm	67
Fluorochroming of Yeast Cells with Acridine Orange ..	71
Technique of Fluorochroming Yeast with Acridine Orange	72
Fluorochroming of Living and Dead Yeast Cells (<u>S. cerevisiae</u>)	72
Quantitative Studies Using a Colorimeter	74
pH Dependence of Acridine Orange Fluorochroming of Yeast	77
Microcultivation Experiments with Vitally Fluorochromed Yeast Cells	77
Investigation of the Effect of Acridine Orange on Yeast Cells	79
Fluorochroming of <u>Mucor</u>	81
Acridine Orange Fluorochroming of Bacterial Suspensions	83
Acridine Orange Fluorochroming of Bacterial Suspensions as a Rapid Laboratory Method	85
Acridine Orange Fluorochroming to Differentiate Between Living and Dead Bacterial Cells	89
Vital Staining of Bacteria with Acridine Orange	99
Applicability of the Acridine Orange Method to Various Bacterial Forms	101
Observations on Gram-Negative Bacteria	102
Observations on Gram-Positive Bacteria	107
<u>Streptococcus pyogenes humanus</u>	113
<u>Sarcina lutea</u>	113
Various Species of Bacilli	113
<u>Azotobacter chroococcum</u> and <u>Azotobacter beijerinckii</u> ..	115
<u>Mycobacterium tuberculosis</u>	117
Observations on <u>Nosema bombycis</u> <u>Nägeli</u> and <u>Nosema apis</u> <u>Zander</u>	119
CELL PHYSIOLOGY OF BACTERIA	119
Introduction	119
Plasmolysis	120
Effects of the Medium	123

Resistance Physiology of Bacteria	126
Local Degenerative Phenomena in Bacterial Proto- plasts	129
FLUORESCENCE-OPTICAL ANALYSIS OF SOIL FOR MICROORGANISMS	131
Introduction	131
Fluoroscopic Analysis of Soil Bacteria Using the Ac- ridine Orange Fluorochroming Method	132
Acridine Orange Fluorochroming of Soil	133
Qualitative Results to Date with Acridine Orange Fluorochroming of Soils	135
Fluoroscopic Analysis for Chlorophyll-Containing Microorganisms	139
Fluorescence-Microscopic Observation of Bacterial Spores in the Soil	140
Brilliant Sulfoflavin-Coriphosphine Staining for Elective Visualization of Bacterial Spores in the Soil	140
Fluorescence-Microscopic Counting of Living Bacteria in the Soil	141
Suggestions for Microbiological Assays of Soil Sam- ples with the Fluorescence-Microscopic Method ...	148
The Problem of Autochthonous Bacterial Flora in the Soil	149
BIBLIOGRAPHY	151
PLATES I, II, III	164
INDEX	169

FOREWORD

Fluorescence microscopy has become increasingly important in recent decades in biomedical research. This method has shown itself to be suitable both in theoretical matters and, especially, in the practical aspects of microbiology.

Work with the fluorescence microscope is tied up with numerous experiences. I have therefore decided to put together my own experiences in an orderly form in order to promote the use of fluorescence microscopy in biology and medicine. As a result of the war it has become quite difficult to obtain access to the individual widely scattered publications and I am convinced that through this compilation, which was supplemented with still unpublished results, the field of fluorescence microscopy can only be advanced.

I should like to thank the publisher for bringing out my book at this very difficult time. The critical reader should regard this monograph as an attempt to promote the cause but in view of the situation now prevailing in the literature, it can lay no claims to completeness. Since I shall attempt to achieve this in the next edition, I would be grateful if my readers called my attention to any gaps.

I offer this monograph to the public in the hope of serving the cause of international cooperation among scientists.

Hannover, 15 March 1958

S. Strugger

INTRODUCTION

Progress in the field of microbiology along with other technical innovations is tied up with the perfection of the microscope. When Abbe introduced an illumination device and oil immersion, he greatly facilitated the discovery of a variety of causative agents of disease, e.g., the tuberculosis bacillus by Robert Koch. Weigert's method of staining fixed bacterial smears (1875) and Gram's stain (1884) were pioneering efforts in bacteriology. In the future the improvement of microscopic research technique and refinement of staining procedures together with the introduction of the electron microscope would have a decisive influence on microbiological research.

When A. Kohler built his famous ultraviolet microscope (1901), he created in principle the first fluorescence microscope. As early as 1904 he referred to the possibility of using his ultraviolet microscope to investigate fluorescence phenomena in microscopic objects. Lehmann (1910, Zeiss-Jena) reported on a filter for ultraviolet rays and its application and he built a fluorescence microscope (1913, 1914). Reichert (1911) and Heimstedt (1911) at about the same time as Lehman designed a useful fluorescence microscope in Vienna. The use of fluorescence microscopic technique in studying biological problems was gradually able to develop after these first instruments for observing fluorescence phenomena in microscopic preparations became available. Progress was very slow at first. Stubel (1911) reported on the primary fluorescence of animal tissue in ultraviolet light. Wasitzki (1913) recognized the usefulness of the fluorescence microscopic method in pharmacology. Willschke (1914) reported on primary chlorophyll fluorescence in plant physiology. Provazek (1914) was the first to conduct observations on microbiological objects with respect to primary fluorescence. Secondary fluorescence in spirilla and protozoans was discussed in the work of P. Metzner (1919, 1924), who was concerned with analyzing the effect of photodynamic matter and the relationship between photodynamic phenomena and phototaxis. Kaiserling (1921) was the first to report on primary fluorescence in bacteria. Arloing, Policard, and Langeran (1925), Gassul and Zolkevic (1927), Lasseur, Dupaix, and Lecaille (1931) also dealt with this phenomenon. The works of Jancso (1932) and Fischl and Schwenk (1932) are a landmark in the use of fluorescence microscopy in bacteriology because they discuss for the first time the intravital fluorochroming of trypanosomes and try to make this new method of chemotherapeutic research serviceable. Hegedus (1938) was able to determine the intravital uptake of trypaflavine on trypaflavine-containing media by bacteria growing there. Hirt (1939) states that he was able to determine the fluorochroming of trypanosomes, spirochetes, and bacteria after injecting experimental animals with trypaflavine. This first period of fluorescence microscopic research draws to a close with the summary account of Haitinger (1938). It is Haitinger's great merit to have provided through his countless special studies the first comprehensive treatment of fluorescence microscopy

techniques and in sweeping fashion laid down the basic principles in a clear form.

The second new period of fluorescence microscopic research in the service of microbiology is marked by the works of Hagemann (1937, 1938) and the studies of Strugger (1941, 1942). While Hagemann made the technique of fluorochroming of fixed bacterial smears available to bacteriologists, he made an extensive study of fluorochrome treatment of living protoplasm and introduced vital fluorochroming of bacteria as a new bacteriological procedure.

In the course of this development it was shown that the fluorescence microscope has more significance for microbiological research than simply as a supplement to normal microscopic investigation of micro-organisms. The advantages of fluorescence microscopy have such great practical value and the new methodological possibilities resulting from this microscopic staining and observation technique are so important for the further development of microbiology that we deem it worthwhile to summarize the principles established to date in order to make more comprehensible and accessible the principles that evolved with further progress in the field.

Agreeing with Hamperl (1943) we must point out here that fluorescence microscopy is not intended gradually to displace bright-field microscopy. On the contrary, because of its special capabilities it will supplement the existing microscopic research methods and be used where it is specially suited. As a practical tool fluorescence microscopy will inevitably have to be considered by microbiologists, and, in fact, it can be safely said even now that microbiology will make great progress as a result of further development of fluorescence microscopic observation and staining technique.

PRINCIPLES OF FLUORESCENCE MICROSCOPY

Fluorescence

A body can be excited by a variety of external influences to emit fluorescent radiation. For example, a body becomes self-luminous when heated to a certain temperature. Our light sources generally operate according to this principle. The phenomenon of light radiation from a heated body is known in physics as thermoluminescence. Also in some chemical oxidation reactions cold light phenomena appear which we group together under the collective name of chemoluminescence.

In the case of a body becoming its own radiator upon being struck by light rays we have the phenomenon of photoluminescence. This can be divided into two closely related phenomena. If the excited radiation of the body persists only as long as the exciting light strikes the body, the excited light is called fluorescence. If, however, the radiation

of the body continues for some time after the exciting light is no longer present, the phenomenon is called phosphorescence.

In theory, fluorescence and phosphorescence cannot be clearly separated for research has shown that even in the case of fluorescence, an extremely brief afterglow can be demonstrated experimentally. Nevertheless, the separation is worthwhile for practical purposes.

There are regular correlations between the wavelength of the exciting light and the wavelength of the emitted fluorescence. Stokes' law states that in general the exciting light has to have a shorter wavelength. For example, a blue fluorescent light can be practically achieved only with an ultraviolet exciting light. On the other hand, a green, yellow, or red fluorescent light can also be produced by a visible, blue exciting light. Hence, to obtain a fluorescence and to test experimentally fluorescence phenomena in bodies, it is best to work with filtered ultraviolet as an exciting light, whereas fluorescence excitation by blue light for special tasks, especially in practical execution of fluorescence microscopy, is highly important if the center of gravity of the fluorescent light under study lies in the green, yellow, or red.

In view of the obviously complicated state of affairs, it would be very difficult and premature to attempt to elucidate the fluorescence phenomenon theoretically in all its ramifications. For simple cases, as with a number of gases which produce simple line fluorescence spectra, the phenomenon can be reduced to a line resonance (sodium vapor). Line resonance occurs as a result of electrons bursting from an inner shell into an outer one due to exciting light radiation whereby it absorbs a certain amount of energy. When the electrons recoil, part of this energy is again given off in the form of fluorescence radiation. Plate I, Fig. 1 shows a series of fluorescence spectra of various substances. These spectra consist of lines and broad bands in different wavelength regions. The simple explanation of fluorescence given above for gases is not applicable to the complicated spectra of polyatomic fluorescing substances. In operation here, besides line resonance, are vibrations of the atoms against one another in the molecule association, molecule rotation, and the reciprocal influences of the electrical fields from molecule to molecule and they show the very complicated fluorescence radiation (Pringsheim, 1928; Danckwortt, 1940; Déhére, 1933, 1937, 1938).

The extraordinarily high sensitivity of the optical identification of fluorescing substances with the help of fluorescence analysis is particularly important for biological application of the fluorescence phenomenon. Unlike the diachromes (dyes in the true sense), fluorochromes (fluorescent dyes) can be identified in extremely diluted form by the fluorescent light they emit. Table 1 shows the clear superiority of fluorescence analysis in a comparison with some diachromes. The table was not prepared perhaps with the use of particularly sensitive methods, but it does show the results of test tube tests determined by gross inspection. The fluorochromes were judged under a quartz lamp.

Table 1

(1) DIACHROME							
(2) Farbe	(3) Konzentration in Leitungswasser						
	1 : 100	1 : 1000	1 : 10000	1 : 100000	1 : 1 Mill.	1 : 10 Mill.	1 : 100 Mill.
(1) Methyl- blau	(5) tief dunkelblau	(5) tief dunkelblau	tief dusch- scheinend blau	(7) hellblau	(8) Spur blau		
(9) Neutral- rot	(10) dunkelrot	(11) rubinrot	(12) leuchtend hellrot	(13) blaßrot			
Methyl- orange	(14) dunkel- orange	(15) hell- orange	hell- orange- gelb	(16) hell- orange- gelb	(18) Spur	-	-
(19) Lichtgrün	(20) tief dunkel- grün	dunkel- scheinend dunkelgrün	(22) hellgrün	(23) blaßgrün	-	-	-

1 cm Reagenzglasdicke (24)

1 - Diachrome; 2 - Dye; 3 - Concentration in tap water; 4 - Methylene blue; 5 - deep dark blue; 6 - deep transparent blue; 7 - light blue; 8 - Trace of blue; 9 - Neutral red; 10 - dark red; 11 - ruby red; 12 - luminous light red; 13 - pink; 14 - dark orange; 15 - light orange; 16 - light orange-yellow; 17 - pale orange-yellow; 18 - Trace; 19 - Light green; 20 - deep dark green; 21 - transparent dark green; 22 - light bluish green; 23 - pale green; 24 - 1 cm test tube thickness

(19) FLUOROCHROME							
(18)	(1) Konzentration in Leitungswasser						
	1 : 100	1 : 1000	1 : 10000	1 : 100000	1 : 1 Mill.	1 : 10 Mill.	1 : 100 Mill.
Acridin- orange	(2) kupfer- rot	(3) orange- rot	(4) gelb- grün	(5) grün	(6) hell grünlich- grau	(7) hell grünlich- grau	(8) Spur
Natrium- fluorescein	(9) grün	(5) grün	(5) grün	(5) grün	(5) grün	(10) hell graugrün	(10) hell graugrün
(11) Natrium- oxypyren- trisulfat	(5) grün	(5) grün	(5) grün	(5) grün	(5) grün	(17) grünlich blau	(5) grün
(12) Brilliant- sulfon- flavin	(13) gelbgrün	(13) gelbgrün	(13) gelbgrün	(13) gelbgrün	hell gelbgrün (14)	(15) gelbgrün	(16) Spur gelbgrün

1 - Concentration in tap water; 2 - copper-red; 3 - orange-red; 4 - yellow-green; 5 - green; 6 - light greenish gray; 7 - light greenish gray; 8 - Trace; 9 - Sodium fluorescein; 10 - light gray-green; 11 - Sodium oxypyrene trisulfate; 12 - Brilliant sulfonflavin; 13 - yellow-green; 14 - light yellow-green; 15 - yellow-gray; 16 - Trace of yellow-gray; 17 - greenish blue; 18 - Dye; 19 - Fluorochrome

The Fluorescence Microscope (FM)

While the normal bright-field microscope achieves resolution of a preparation by light absorption and refraction and thus reveals the structure of cells and tissues, the FM works on a fundamentally different principle. When an absolutely nonfluorescent preparation is examined in the FM, the field of view is completely dark. On the other hand, when substances capable of fluorescing are in the field of view, the places in the preparation capable of fluorescing become independent sources of light due to irradiation with exciting light of the shortest possible wavelength, whereas the places incapable of fluorescing remain dark. The light source for the bright-field microscope therefore lies outside of the microscope in the illuminating device, whereas in the FM the fluorescent places in the preparation themselves become the light source. The function of illumination of the preparation with the exciting light is simply to excite the preparation to self-illumination.

The FM is so designed, therefore, as to use a physically suitable exciting light to produce the strongest possible fluorescence in a preparation, with the exciting light quantitatively destroyed by an absorption filter before it leaves the eyepiece. Thus only the excited fluorescent light in the preparation reaches the eye of the observer and maximum resolution of the nonfluorescing part of the field of view is achieved.

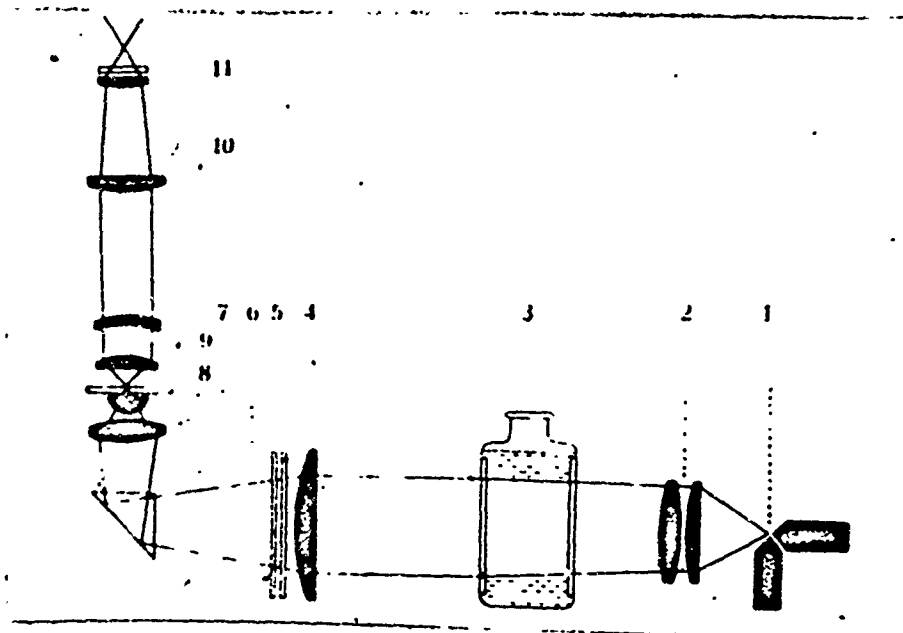


Fig. 1. Path of rays in a simple fluorescence microscope.
Details in the text.

* The above diagram (Fig. 1) shows the path of rays in a simple FM. 1 is the light source for emission of the exciting light. Here it is an arc lamp which emits strong light in the entire spectral region. The condenser lens 2 forms a beam of light which passes through the copper sulfate cuvette 3 and is there freed from the shortwave part of the spectrum. 4 is a converging lens and 5 are the absorption disks for the final filtering of the exciting light. The light then strikes the prism or mirror 6 of the microscope from which it is reflected into the illuminating device. The latter concentrates the light precisely in the field of view of the preparation 8, where fluorescence is excited. The fluorescence and excess exciting light pass through the objective 9 and eyepiece 10. In front of the eyepiece is the absorption filter 11 which quantitatively absorbs the exciting light and permits the longer wave excited fluorescent light alone to pass through unchanged.

In practice two types of illumination are used as exciting light: (1) Blue-light, wavelength 4800-3000 Å, (2) Ultraviolet-light < 3000 Å. Accordingly, the FM is called either a blue-light or ultraviolet-light FM.

To put together and test a FM in the laboratory, the following general guide lines should be kept in mind. Only a plane mirror should be used. The diaphragm of the illuminating device must always remain completely open. The height of the device must be so adjusted that the smallest possible central field of view is maximally illuminated. Normal objectives 3 and 5 can be used if they are free from natural fluorescence. As an immersion objective one can use an objective with the highest possible aperture, e.g., Zeiss apochromatic lens and 1/12 oil immersions. Special care must be taken to ensure that the lenses are free from fluorescence. The strongly fluorescent cedar oil cannot be used as immersion oil. Either the fluorescence-free immersion oil manufactured by the optical firms of Zeiss and Reichert or maximally pure liquid paraffin is used. Ordinary Huygen or photoocular eyepieces are used.

The following practical suggestions may be of value in setting up a FM. Pulverized anthracene (test preparation) is thinly spread on a slide in a drop of liquid paraffin and covered with a cover glass so that a thick anthracene suspension results. Anthracene is known to have a very strong blue-green fluorescence. This preparation is placed on the stage and focused under weak magnification (objective 3). The lamp and mirror position and height of the condenser are adjusted until the maximum possible brightness and uniformity of the anthracene fluorescence in the field of view is achieved. With this apparatus one can proceed to test successfully a FM, especially suitable combinations of filters.

* Metzner (1926, 1930) and Haitinger (1930) discuss ways of using simple materials to make one's own FM.

The Blue-Light Fluorescence Microscope

Any well built laboratory microscope can be readily converted into a workable blue-light FM. The regular mirror of the microscope can be used as a reflector. The normal illuminating device consisting of glass lenses is fully penetrable by the blue light. The following are essential: (1) Choice of a suitable light source for the exciting light, (2) Choice of suitable filter combinations (exciting light filter and eyepiece absorption filter).

Add. 1. The principal rule is to use the strongest possible light source to excite fluorescence in the preparation. Ordinary daylight or makeshift microscope lamps are therefore completely out of the question as light sources. An easily centered low-voltage lamp with a converging lens (e.g., Leitz, Reichert, Zeiss) is suitable when the requirements for brightness are modest. Keller (1939) in cooperation with Reichert developed the low-voltage Lux FNI for blue-light fluorescence microscopy in which the appropriate blue filters are already mounted. This lamp produces a moderately bright but quite even light that is adequate for rather large microbiological objects and fluorochromed bacterial smear preparations. Stronger light sources must be used if the requirements are higher. Simple microscope-carbon arc lamps are particularly suitable for this purpose. They produce a light that is sufficiently strong for all bacteriological-fluorescence research purposes. It is obvious that these arc lamps have to have condenser lenses so that a sharply defined, maximally parallel beam falls on the microscope mirror. Mercury-vapor lamps, known to be very rich in blue light, are suitable for the highest requirements. A converging lens made of glass must be introduced before this light source is used. Suitable burners are the S 100 of the Hanau Quartz Lamp Company and even better a 40 w or 60 w mercury vapor maximum pressure lamp (Osram and Phillips), which has the advantage of possessing an extremely bright, almost punctiform light source. This maximum pressure lamp is the best light source for fluorescence microscopy (Gohde, 1944).

Add. 2. A suitable filter for the exciting light must be chosen with particular care. It is best tested with the spectroscope. Heat radiation has to be removed first. This is done by inserting a copper sulfate cuvette. A 2-5% solution of copper sulfate in distilled water in a 3-4 cm thick layer is sufficient to absorb the heat radiation and most of the red light. The exciting light must then be sent through a blue filter which permits only pure blue light to pass through (Table 2). The correct blue filters are best determined when a spectroscope held against the filtered light permits only a more or less broad blue spectral band to be distinguished. Table 2 lists several tested filter combinations for blue-light fluorescence microscopy.

Table 2

Zeiss blue-light disks from a large FM (2 disks). 5% copper sulfate solution (3-5 cm).
 Reichert blue-light disks (2 disks) mounted in a Lux FNI and in a Reichert FM.
 Copper oxide-ammonia solution, conc. in kuvette. 2-5 cm.
 5% copper sulfate solution (3 cm) and 2 BG-3 disks (1 mm each).
 2 BG-12 disks (1 mm each) and 5% copper sulfate solution (2-3 cm).
 BG 12 (1½ mm) and BG 3 (1 mm) and 5% copper sulfate solution (2-3 cm).
 BG 1 (2 mm) and BG 12 (1½ mm) and 5% copper sulfate solution (2-3 cm).
 BG 1 (2 mm) and BG 3 (1½ mm) and 5% copper sulfate solution.
 Saturated copper oxide-ammonia solution (2½ cm) and 5% copper sulfate solution (2½ cm).

Note: All the BG disks are made by Schott & Gen., Jena. The mm and cm figures refer to the thickness of the layer.

The absorption filter is without doubt one of the most important parts of the FM. Its function is to absorb the excess exciting light before it reaches the observer's eye and at the same time permit the excited fluorescent light to pass through as little changed as possible. The absorption filter is chosen in the following way. The exciting light source is centered on the microscope. The blue filters are the first to be eliminated thereby. If the centering occurs in the total beam of the microscope, the blue filters are inserted in between. Without an absorption filter the strong blue exciting light leaves the eyepiece. Now the absorption filters to be chosen are best tested empirically. Perhaps 1 mm thick orange glass disks can be considered. That absorption filter is suitable which can completely shut out the exciting light for the eye of the observer in this procedure so that the field of view appears as dark as possible. However, the fluorescent light must be able to come through largely unchanged.

Testing with the spectroscope is also worthwhile. For this purpose the exciting light source with its beam is placed against the eye of the observer. After the copper sulfate cuvette and exciting light blue filter are connected, the spectroscope is directed toward the beam of light and a bright but blue light is obtained in the spectroscope. If the absorption filter being tested is suitable for the blue filter combination in question, no visible light should be seen any longer in the spectroscope after the absorption filter is placed in front of the light opening of the spectroscope. Finally, it is necessary to make a test with the fluorescent anthracene preparation, which makes it possible to judge any possible change in the fluorescent light due to a much too powerful absorption filter. The appropriate absorption filter must be first found in this way for every exciting light source and for every exciting light filter combination. Table 3 lists some of the absorption filters that proved to be very good in my laboratory work.

Table 3

(1) Reichert Speerfilter Nr. 8007	
OG 1 (1 mm)	} Schott
OG 1 (2 mm)	
OG 4 (1 mm)	

1 - Reichert absorption filter No. 8007

The simplest makeshift blue light FM uses a low-voltage lamp as a light source. Only blue filters are used to filter the light. There is no need to filter the heat. The combinations tested are listed in Table 4.

The Lux FNI (Reichert), Ortholux, Panphot or Ultraphot devices are are especially practicable because of their good centering.

This setup can be temporarily used with success to observe natural and secondary fluorescence in tissues under low magnification and to observe larger microorganisms. Heavy-duty service is not to be expected and we can only warn against using such an instrument for scientific bacteriological research.

If a more powerful illuminating device is desired, use should be made of a microscope arc lamp, such as that manufactured by optical firms for dark-field investigations and for microphotographic purposes, as a light source. In this case a copper sulfate heat filter has to be placed between the light source and blue filters. The combinations of filters listed in Table 5 stood the test of practice.

This setup is already useful for microbiological investigations. Any good laboratory microscope can be employed as the microscope. For critical investigations of still unresolved matters, we unhesitatingly recommend the use of a heavy-duty FM, which itself can also be built as a blue-light heavy-duty instrument with the help of a mercury-vapor high-pressure lamp and any good research microscope.

I am very grateful to Dr. Krefft for letting me have his Osram maximum-pressure lamps. I tested them very thoroughly in 1943, 1944, 1945, and 1946 and found them to be the best sources of light for fluorescence microscopy.

The filter combinations tested are presented in Table 6.

Table 4

(1) Erregelichtfilter	(2) Absorptionsfilter
(3) Reichert Blaufilter, 2 Scheiben Reichert Blaufilter, 2 Scheiben Reichert Blaufilter, 2 Scheiben	Reichert 8807 (HG 1 (1 mm) (HG 1 (2 mm)
(4) Kupferoxyd-Ammoniak, konz. (2 1/2 cm) Kupferoxyd-Ammoniak, konz. (2 1/2 cm) Kupferoxyd-Ammoniak, konz. (2 1/2 cm)	Reichert 8807 (HG 3 (1 mm) (HG 4 (1 mm)
(5) 2 mal BG 3 (Schott) (1 mm) und 5%iges Kupfersulfat, (2 1/2 cm) 2 mal BG 3 (Schott) (1 mm) und 5%iges Kupfersulfat, (2 1/2 cm) 2 mal BG 3 (Schott) (1 mm) und 5%iges Kupfersulfat, (2 1/2 cm) 2 mal BG 12 (Schott) (1 mm) und 5%iges Kupfersulfat, (2 1/2 cm) 2 mal BG 12 (Schott) (1 mm) und 5%iges Kupfersulfat, (2 1/2 cm) BG 12 (Schott) (1 1/2 mm) und BG 3 (Schott) (1 mm) und 5%iges Kupfersulfat, (2 1/2 cm) BG 12 (Schott) (1 1/2 mm) und BG 3 (Schott) (1 mm) und 5%iges Kupfersulfat, (2 1/2 cm) BG 1 (Schott) (2 mm) und BG 12 (Schott) (1 1/2 mm) und 5%iges Kupfersulfat, (2 1/2 cm) BG 1 (Schott) (2 mm) und BG 3 (Schott) (1 1/2 mm) und 5%iges Kupfersulfat, (2 1/2 cm) BG 1 (Schott) (2 mm) und BG 3 (Schott) (1 1/2 mm) und 5%iges Kupfersulfat, (2 1/2 cm)	Reichert 8807 OG 1 (1 mm) (HG 4 (1 mm) Reichert 8807 (HG 1 (1 mm) Reichert 8807 (HG 1 (1 mm) Reichert 8807 Reichert 8807 (HG 1 (1 mm)

Sugger Fluoreszenzmikroskopie

1 - Exciting-light filter; 2 - Absorption filter; 3 - Reichert blue filter, 2 disks; 4 - Copper oxide-ammonia, conc.; 5 - 5% copper sulfate

THE UV-Light Fluorescence Microscope

A UV-light FM cannot be readily put together in the laboratory. It must be built for this specific purpose as a special microscope. The large UV-light FM of the Zeiss and Reichert optical firms can be converted through simple switching of the light filter to a heavy-duty blue-light FM. Thus the instruments can find quite universal application. The greatest research difficulties can be overcome with these instruments.

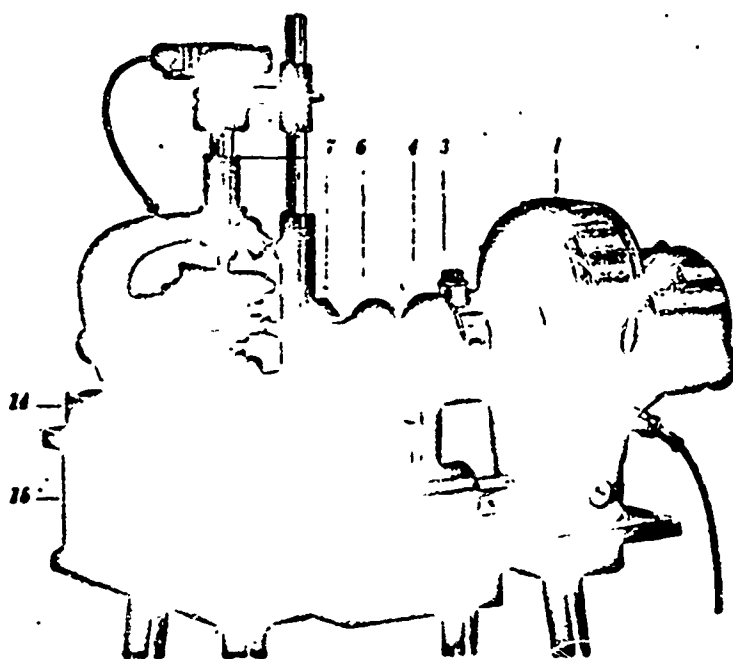


Fig. 2. General view of the large Zeiss (Jena) fluorescence microscope. The numbers correspond to those in Fig. 3.

We must first mention the large Zeiss FM which operates with a 10 amp carbon-arc lamp and special carbon as a light source. It has turned out to be best for research purposes. Zeiss recently put out a special lamp housing in order to make mercury-vapor maximum-pressure lamps (Osram) usable with this apparatus. Fig. 3 shows a diagrammatic section through this apparatus. 1 is a 10 amp arc lamp, which is best powered with a 120 v direct current. The light streaming from the crater of the arc lamp is collected by two quartz collector lenses (2) and led in a weak divergent beam into the copper sulfate cuvette (3). This cuvette is filled with a 2-3% copper sulfate solution. 4 is an iris diaphragm which helps to limit the beam. The beam is made convergent

by the quartz lenses (5) and passes through the exciting-light filter (6). These are interchangeable so that it is easy to switch from blue-light to ultraviolet-light illumination. The light then strikes the position of the mirror in a completely reflecting rectangular quartz prism (8) from which it passes upward into the lenses of the quartz condenser (10). The exciting light is concentrated in point fashion in the object under study so that the strongest possible fluorescence is excited. 11 is the objective, 12 the eyepiece, and 13 the absorption filter, which likewise must be readily interchangeable. The apparatus is centered by shutting the iris diaphragm at 4 and by placing a uranium glass (9) on the quartz prism between 8 and 10. The quartz condenser itself is set in a centering frame so that in case of low magnification good centering with the help of the anthracene preparation is possible with direct microscopic observation. The quartz condenser has two interchangeable upper pieces. The one with the larger front lens diameter is used between the condenser lens and slide without immersion fluid, whereas the upper piece with the smaller front lens diameter can only be used as an immersion condenser and is suitable for the highest magnifications.

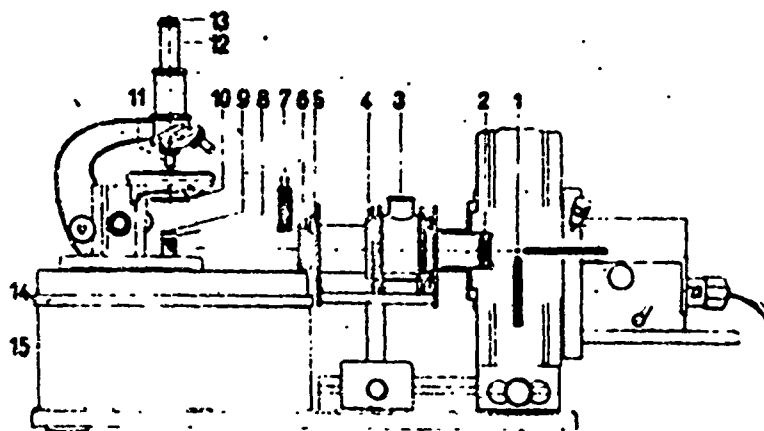


Fig. 3. Schematic longitudinal section through the large Zeiss fluorescence microscope. 1 - arc lamp, 2 - double-lens posterior member of the quartz collector, 3 - copper sulfate cuvette, 4 - iris diaphragm, 5 - anterior member of the quartz collector, 6, 7 - two UV filters to absorb visible light for UV fluorescence microscopy, two blue-light filters for blue-light fluorescence microscopy, 8 - quartz prism in the place of the mirror, 9 - uranium glass to center the beam, 10 - quartz condenser, 11 - objective, 12 - eyepiece, 13 - eyepiece absorption filter, 14 - footplate, 15 - wooden case.

For UV fluorescence microscopy the two inside filter disks (6) are individually inserted and a Euphos filter is placed on the eyepiece as an absorption filter. The firm produces two blue disks (the two outside disks 7) for blue-light fluorescence microscopy. Reichert's absorption filter No. 8007 is used in working with blue light.

The large Reichert FM has turned out to be best as a second FM. It operates with a mercury-vapor lamp (Hanau S 100) and is also excellent for particularly delicate bacteriological studies. By changing the filter combinations supplied with the instrument one can use it for both UV and blue-light fluorescence microscopy.

Practical Use of the UV and Blue-Light Microscopes

From the physical standpoint the UV FM is the finest instrument available for exciting fluorescence in a microscopic preparation. The fluorescent colors of the entire visible spectrum can be clearly observed with this instrument; so too the blue fluorescence of a biological object. Therefore, the UV-light FM should always be used whenever it is important to maintain the spectral composition of the fluorescent light as complete and as unchanged as possible. It is quite indispensable especially for observation of blue fluorescence, which appears quite often as primary fluorescence in organic material. Its disadvantages are that the intensity of the fluorescence is somewhat less than that encountered in blue-light fluorescence microscopy and strong irradiation of living objects with concentrated UV light injures them. These disadvantages can be overcome by using the blue-light FM. Blue fluorescence cannot be seen with this instrument owing to total blue absorption by the absorption filter. Only green, yellow, and red fluorescent colors are reproduced more or less realistically. The spectral composition of the fluorescent light will undergo greater or lesser changes, depending on the characteristics of the absorption filter used. This physical disadvantage is not too serious, however, in the case of most investigations. Instead, the blue-light FM has a significantly greater light intensity and is particularly suited for delicate cytological research. Another inestimable advantage of the blue-light FM is that the concentrated blue exciting light does relatively little damage to a living biological object.

The following rules should be followed in using UV and blue-light fluorescence microscopy:

(1) If it is important for the investigator to determine physically and perfectly blue fluorescent colors in a biological object as well as to observe the spectral composition of the fluorescent light objectively, only a heavy-duty UV FM will do the job.

(2) On the other hand, if green, yellow, and red fluorescent colors are to be observed in a living object, the blue-light FM is preferable in every case.

Owing to the very great intensity of irradiation of the object, it is recommended in working with the FM on either living or nonliving objects that a single place on the preparation should not be irradiated too long. Disturbances are often evident in a living object within 10 seconds of use of a powerful instrument while nonliving objects can fade. It is desirable, therefore, to move the preparation constantly in order to prevent such disturbances and thereby obtain an objective judgment on the condition of the undisturbed preparation.

FLUOROCHROMING OF FIXED BACTERIAL SMEAR PREPARATIONS

Introduction

The use of organic dyes is one of the most important techniques in bacteriology. It permits optical differentiation of fixed bacterial smears in such a way that the individual cells clearly stained contrast with the bright background. All the usual bacteriological investigations carried out in medical-bacteriological laboratories employ this smear technique.

By fluorochroming fixed bacterial smears Hagemann (1937) introduced a new method for microscopic preparation of bacteria in a fixed condition that immediately aroused great interest owing to its manifold advantages. This method "stains" bacterial smears with fluorescing substances so that when the preparation is examined in the FM, the individual bacterial cells light up in bright fluorescent colors against a completely dark background. This optical differentiation is highly advantageous. It facilitates observation of the bacteria which emit independent fluorescent light. The ability to find germs scattered throughout the preparation is decidedly improved by the method. Moreover, smear preparations can be "stained" more quickly than by the procedures currently in use so that there is a considerable savings of time. Therefore, it cannot be doubted that the fluorochroming of fixed smear preparations will not only maintain its place in the bacteriological laboratory, but will become even more popular in the future.

Definitions and Characteristics of Fluorochromes

A fluorochrome is a substance which when in solution can impart fluorescence to a nonfluorescing substance. This is possible only if the fluorochrome is bound in some form with the nonfluorescing substance. This binding is called storage. All fluorescence phenomena which are obtained by treating originally nonfluorescing substances with fluorochromes are called secondary fluorescence phenomena. These are opposed to primary fluorescence phenomena, i.e., substances which fluoresce naturally. The treatment of a nonfluorescing preparation with fluorochromes in order to impart secondary fluorescence is called fluorochroming.

Fluorochroming involves in principle the same procedures as for the usual staining of a preparation. The fluorochromes are stored in the bacterium by chemical or physical forces and held fast. The use of dyestuffs which appear stained in transmitted light is called diachroming, in contrast with fluorochroming, and the bright-field dyestuffs themselves are called diachromes.

It has been shown that a thorough knowledge of the theory of fluorochroming is of particular value for practical purposes. Further progress in the use of fluorescence microscopy in the fields of micro- and microbiology is conceivable only on the basis of a solid knowledge of the underlying theory.

The number of fluorescing substances is extremely large. There are surprisingly few inorganic compounds that fluoresce, e.g., the uranium compounds. Most fluorescing substances are to be found among the organic compounds. These fluorochromes belong to a great variety of groups of organic compounds (Danckwortt, 1940; Déhére, 1933, 1937; Haitinger, 1938). From the physical-chemical standpoint, however, they can be clearly divided into three groups: (1) cathodic fluorochromes, (2) anodic fluorochromes, and (3) electroneutral (lipophilic) fluorochromes.

Add. 1. The cathodic fluorochromes are more or less strongly dissociated in aqueous solution. The cations are the carriers of the fluorescence phenomenon. They behave, therefore, like the cathodic (basic) diachromes.

Goppelsröder's capillary test (1901) for detection of the cathodic or anodic nature of fluorochromes and diachromes, as recently improved by Liesegang (1943)*, has proved to be best in laboratory practice. An aqueous solution of the fluorochrome to be tested (pH between 3 and 7) is pipetted onto filter paper. The capillary spread of the fluorochrome on the filter paper is promptly examined under a quartz analytic lamp. A blue-light lamp can also be used here in the case of non-blue fluorescing fluorochromes. If the particular fluorochrome is cathodic, the fluorescing part of the solution takes longer to spread than does the solvent water. As a result, one can observe on the filter paper in the spread drops a strongly fluorescing nucleus with a more or less broad water margin on the periphery that does not fluoresce.

If the dependence of the dissociation on the pH of the aqueous solution is investigated in a cathodic fluorochrome, the curve shown in Fig. 4 generally results. The peak of the dissociation lies in the more or less acid region, while the dissociation experiences a retrogression toward the neutral point and finally drops to zero. Depending on the

*It is my pleasant duty to sincerely thank Prof. R. E. Liesegang for making available to me in a letter his latest method of cross analysis.

chemical nature of the fluorochrome employed, dissociation ceases in the more or less alkaline region. If the minimum is exceeded, the fluorochrome lies molecule-dissolved in the alkaline region in the undissociated and therefore electroneutral base solution. In many fluorochromes no significant change in fluorescence characteristics can be detected on cessation of dissociation since the molecules fluoresce just as intensely as the cations. There are, on the other hand, some cathodic fluorochromes which as cations in the pH region in which they are dissociated show another fluorescent color than they do in alkaline solution as a molecule-dissolved system. Such fluorochromes are then called cathodic fluorescence indicators. Like the color indicators they possess a characteristic transition area. This area is identical with the pH interval in which the dissociation of the fluorochrome becomes practically zero. There are two-color fluorescence indicators which are distinguished by the fact that the fluorescent color of the cation is clearly different from that of the base molecule. By way of contrast, there are one-color fluorescence indicators with which a distinct fluorescence appears either in dissociated state or in molecule-dissolved state, whereas the other form (either the molecule or the ion) does not fluoresce. Table 7 lists some important one- and two-color cathodic fluorescence indicators together with the transition regions.

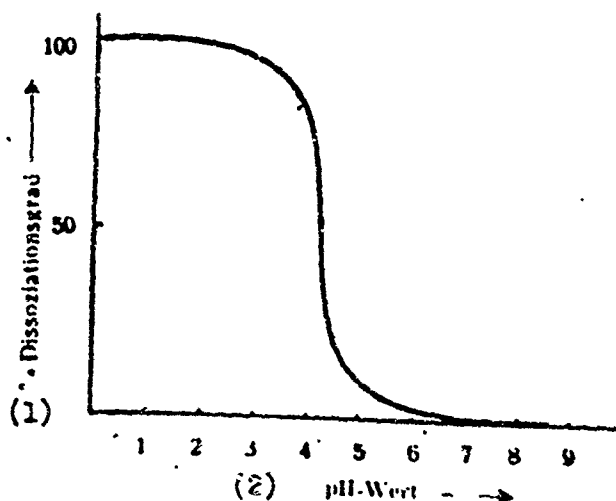


Fig. 4. pH dependence of dissociation in cathodic fluorochromes

1 - % degree of dissociation; 2 - pH value

Table 7

Name	(1) pH-Umschlagsbereich	(2) Fluoreszenzfarbe
Benzollavin	0.4 - 0.7	(3) gelb / grün
(4) Chininsulfat	0.8 - 0.1	(5) hellblau / violett
Acridin	4.8 - 5.0	(6) eisblau / ultramarin
Acridinorange	8.4 - 10.4	(7) schwach / stark laubgrün / gelbgrün
(8) Neutralrot	7.0	(9) farblos / kress
Pyronin	10.0 - 10.3	(10) gelb / ultramarin
(11) Acridinfot	10.0 - 11.0	(10) gelb / ultramarin

1 - pH transition region; 2 - fluorescence color; 3 - yellow/green; 4 - quinine sulfate; 5 - light blue/violet; 6 - ice blue/ultramarine; 7 - weak/intense, leaf-green/yellow-green; 8 - neutral red; 9 - colorless/cress; 10 - yellow/ultramarine; 11 - acridine red

The regular relationship between dissociation and pH value is also paralleled by the liposolubility (solubility in organic phases) of the fluorescing components. For the cathodic fluorochromes it is a general rule that the fluorescing cations have a purely hydrophilic character and are therefore completely liposoluble, whereas the undissociated base molecules are distinguished by a selective solubility in organic solvents. An experiment in extracting pyronine by shaking illustrates this behavior (Table 8).

Table 8

pH	Benzol	(1) Farbuflösung
2.00	+	(2) ultramarinblau 14 pa
3.47	+	(2) ultramarinblau 14 pa
4.99	+	(2) ultramarinblau 14 pa
7.82	+	(2) ultramarinblau 14 pa
10.00	+	(2) ultramarinblau 14 pa
11.80	+	(2) ultramarinblau 14 pa

1 - Dye buffer solution; 2 - ultramarine blue 14 pa; 3 - yellow 2 na

The figures refer to the Ostwald color scale.

Experiments in extraction by shaking are carried out in such a way that graduated buffered fluorochrome solutions are produced between pH 2 and pH 11 and covered with a layer of benzene or toluene in test tubes. The latter are then vigorously shaken and allowed to stand until there is a clear separation between the aqueous phase and the organic phase. They are then examined under a quartz lamp. It is evident from the table that the yellow fluorescing pyronine cations are hydrophilic, whereas the azure fluorescing color base molecules are lipophilic.

The fluorescence colors of many fluorochromes in molecule-dissolved state vary in aqueous and organic solvents, an important phenomenon in fluorochroming practice. For example, the neutral red base fluoresces quite weakly orange-yellow in aqueous solution but intense light green in organic solution. In the scientific production of fluorochromes these relationships must at times be made clear by suitable model tests.

The concentration of the fluorochrome is also significant both for its intensity and color. In many fluorochromes the fluorescence changes with increasing concentration; this is called the "concentration effect" (Strugger, 1940). Thus most substances often fluoresce more weakly in concentrations between 1:100 and 1:1000 than in concentrations between 1:5000 and 1:100,000. The best example of a change in fluorescence color with changing concentration is perhaps acridine orange (discussed in a special section, cf. p.50).

Some basic fluorochromes exhibit a distinct relationship between the fluorescence and electroadsorptive storage of the color cations in electronegatively charged systems. Auramine is one of the best examples of this. This cathodic fluorochrome is dissociated in the entire acid region to pH 9.0. Its cations do not show any specific fluorescence in aqueous solution, whereas in an adsorbed state (e.g., after they are electroadsorptively bound on filter paper) they have a bright, golden yellow fluorescence. Here the fluorescence is not activated until the cations are bound electroadsorptively on a colloidal surface.

Add. 2. Fluorochromes whose fluorescing portions in dissociated state are anions are called anodic fluorochromes. They correspond to the acid diachromes and exhibit similar regularities.

The capillary test with these fluorochromes is carried out as follows. After the aqueous solution of an anodic fluorochrome is dripped onto filter paper, the fluorescing components and the aqueous solvent are seen to spread almost simultaneously under the quartz lamp. The explanation of this behavior is relatively simple. The negatively charged fluorescing anions are not bound electrostatically by the negatively charged fibrous material of the filter paper and can therefore spread by capillary action simultaneously with the water.

Fig. 5 is a curve showing the dependence of dissociation of an anodic fluorochrome on the pH of its solution. The dissociation is maximal in the alkaline to neutral region. Toward the acid side, on the other hand, the dissociation decreases to zero. The pH value at which the dye appears in solution as molecules varies from dye to dye. Also among the anodic fluorochromes there is a number of one-color, less commonly, two-color indicators. Table 9 lists some examples.

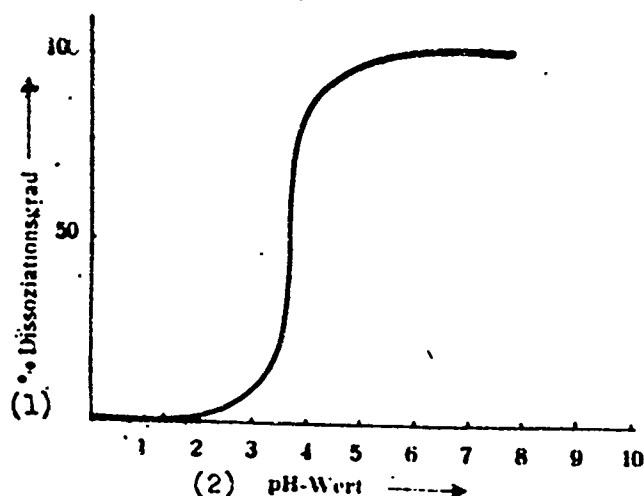


Fig. 5. pH dependence of dissociation in anodic fluorochromes.

1 - % degree of dissociation; 2 - pH value

Anodic fluorochromes are, for the most part, completely lipid-insoluble and they rarely exhibit a pronounced concentration effect.

Add. 3. The third category includes the electroneutral fluorochromes whose dissociation in the entire pH region is so slight that it becomes practically zero. They do not show a significant pH-dependence of dissociation. Since they appear chiefly in molecular solution, they are very selectively lipid-soluble. The most important electroneutral fluorochrome is rhodamine B together with some related substances (Strugger, 1938; Drawert, 1939).

The degree of dispersion is important for all three classes of fluorochromes. While most fluorochromes are molecule or ion disperse, there are also some colloid disperse ones, e.g., primuline. When primuline is diffused into 10% gelatin solution, a distinct phase separation appears. A rapidly diffusing ultramarine blue fluorescing component precedes a very slowly diffusing yellow fluorescing component. It is a question here of a polydispersity which is significant for the fluorochroming of organic structures in that as finely porous systems they can be fluorochromed only ultramarine blue; as coarsely porous, yellow as well.

Table 9

Name	(1) pH-Umschlagsbereich	(2) Fluoreszenz/farbe
Eosin	0.0 - 3.0	farblos - gelbgrün (3)
Aesculin	1.1 - 1.5	indigo - blau (12)
Phloxin	1.4 - 3.0	farblos - gelb (4)
Fluorescein	3.8 - 6.2	farblos - grün (5)
Erythrosin	4.0 - 4.5	farblos - grün (5)
Chininpurpur (6)	4.0 - 5.0	gelb - blau (7)
Chromotropensäure (8)	6.0 - 7.0	farblos - blau (9)
8-Methylumbelliferon	7.0 - 7.2	farblos - blau (9)
Cumarsäure (10)	7.2 - 9.0	farblos - grün (5)
Cumarin	9.5 - 12.0	schwach grün - hellgelb (11)

1 - pH transition region; 2 - fluorescence color; 3 - colorless/yellow-green; 4 - colorless/yellow; 5 / colorless/green; 6 - quininic acid; 7 - yellow/blue; 8 - chromotropic acid; 9 - colorless/blue; 10 - coumaric acid; 11 - pale green/light green; 12 - indigo/blue

It is recommended, therefore, that such diffusion tests in gelatin be taken into account when observing questionable phenomena.

THE THEORY OF FLUOROCHROMING OF BACTERIAL SMEARS

Model Tests

If fixed bacterial cells are to be effectively fluorochromed, the fluorochrome has to be bound either on the cell membrane or on the protoplasts. In view of the generally very delicate structure of the membrane, adherence of the fluorochrome to the fixed protoplasm is of decisive importance for effective fluorochroming of bacterial smears. For this reason we shall discuss first model tests of membrane substances, especially model tests of pure proteins or a protein-lipid system.

The cellulose material of a defatted piece of sterile absorbent cotton is a useful model object for membrane substances. With the help of colorimetric extinction measurements, my co-worker Kolbel (1947) quantitatively determined the pH dependence of the dye storage of cellulose fibers, using the cathodic dia- and fluorochrome auramine in a dye bath lasting 10 minutes. The curve in Fig. 6 shows that the cellulose fibers are scarcely able to store auramine close to pH 2. It is only above pH 2 that a clear-cut rise in storage can be seen; it reaches a maximum at pH 6. 0.4% of the specific weight of the introduced cellulose

is then absorbed from the dye bath. From pH 6.5 the storage again declines. From the standpoint of an electroadsorptive binding of the dye on the cellulose, this curve is to be understood in the following manner. The cellulose is gradually discharged with increasing pH value so that the dye cations can no longer be proportionally adsorbed on its inner surface. With rising pH value, however, the electronegative charge of the cellulose increases and with it the electroadsorptive binding capacity of the dye.

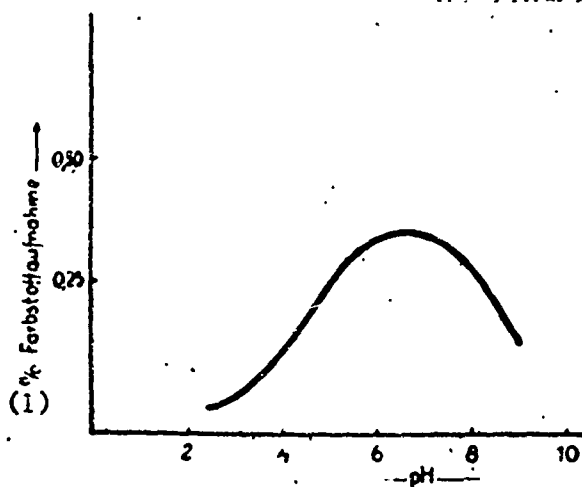


Fig. 6. Colorimetric determination of auramine storage by cellulose fibers in relation to pH value; Concentration of the dye $c = 0.5 \cdot 10^{-3}$ mol/ltr. Ordinate: per cent of dye uptake relative to weight of the colored cellulose (Kolbel, 1947).

(1) % dye absorption.

In the alkaline region, to be sure, the negative charge of the cellulose is big, but the dissociation of the dye decreases rapidly toward pH 9. Consequently, this dissociation retrogression emerges as a limiting factor for the electroadsorption of auramine in the alkaline region. What has been said here about the cathodic auramine applies in principle to all the cathodic fluorochromes with respect to storage on membrane substances. It is quite evident from these model tests that cathodic fluorochromes can best fluorochrome the membrane substances of cells between pH 4 and 8. Anodic dyes, on the other hand, cannot be used within this pH range as membrane fluorochromes.

With respect to analysis of the fluorochroming of fixed bacterial protoplasts, it can be said in general that the protoplasm is a system composed of proteins and lipids. Model tests of pure proteins or protein-lipid mixtures are also suited for elucidation of the dyeing of dead protoplasts.

Proteins are amphoteric and therefore show a peculiar pH dependence. Every protein has a narrow pH range in which the number of dissociated OH ions is the same as the number of dissociated H ions. Such a protein, since it is neither positively nor negatively charged, is called an isoelectric protein. If this isoelectric zone (or IEP) is exceeded or not reached through change in the pH, we are confronted with either an electronegative or electropositive protein. Accordingly, the fluorochroming of proteins with anions or with cations must show a strict, regular pH dependence if the fluorescing ions are electrostatically bound. This correlation can be clearly shown in Drawert's model test (1937) in gelatin with the pair of diachromes acid fuchsin and toluidine blue. Fig. 7 shows the pH dependence of the storage of the anodic acid fuchsin and the cathodic toluidine blue in gelatin. The IEP of the gelatin-protein used lies at pH 4.6. Since gelatin is virtually electroneutral in this pH region, it can store either the acid fuchsin or the toluidine blue. For both dyes, therefore, the storage minimum coincides with the isoelectric zone of the gelatin. If the IEP is not exceeded owing to stronger acidification of the dye bath, increasing storage of the acid fuchsin anions in the gelatin takes place. The gelatin becomes electropositive. If, however, the IEP is exceeded owing to increase in the pH, electroadsorptive storage of the toluidine blue cations in the gelatin increases. The gelatin becomes more and more electronegatively charged. This is the principle underlying the method of determining the IEP with a pair of dyes (Naylor, 1926; Pischinger, 1926, 1927; Drawert, 1937).

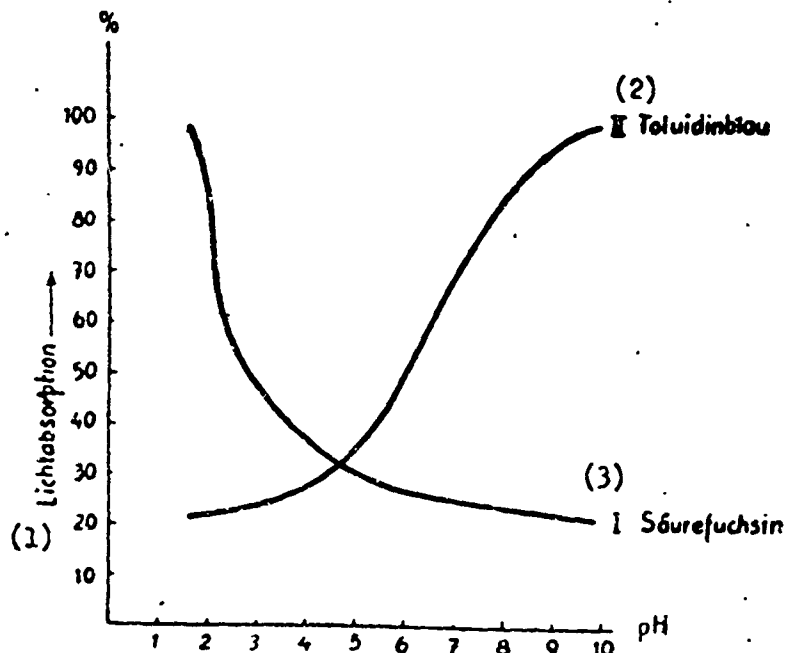


Fig. 7. pH dependence of toluidine blue and acid fuchsin storage by gelatin. Both curves meet in the IEP; the point represents the minimum of storage for both dyes (Drawert, 1937).

1 - light absorption; 2 - II-toluidine blue; 3 - I-acid fuchsin

The absorption of acridine orange by gelatin, as quantitatively determined by Kölbel (1947), fits in well into this pattern (Fig. 8). The IEP of the gelatin used lay between pH 3 and 4. In this range the storage of the cathodic acridine orange in the gelatin is very slight. The maximum (0.6% of the specific weight) is not achieved until pH 4 to 8. Then the storage curve falls again in the alkaline region while the dissociation of the acridine orange declines.

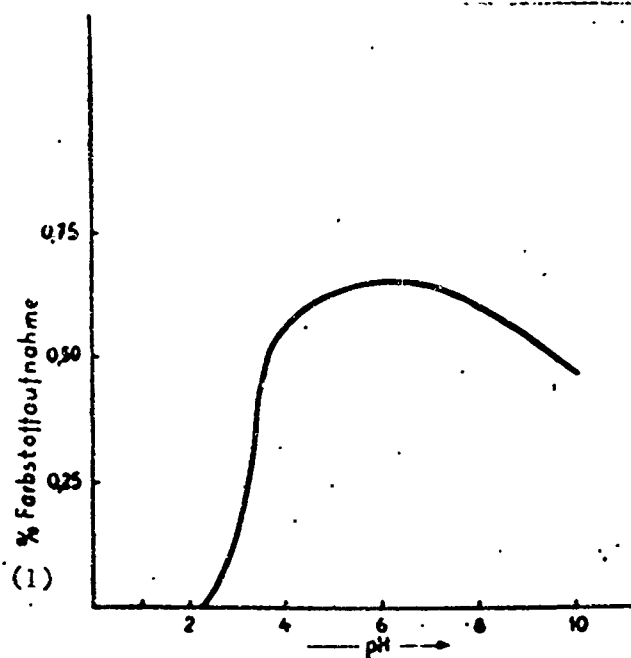


Fig. 8. Colorimetrically determined pH dependence of acridine orange storage in gelatin. Concentration of the dye $c = 0.5 \cdot \text{mol/ltr}$. Dye uptake in per cent of weight of the colored gelatin (Kölbel, 1947).

(1) % dye absorption.

It can be safely deduced from such a model test that amphoteric protein systems must show the storage maximum for cathodic fluorochromes above their IEP; for anodic fluorochromes, under their IEP in the extreme acid region. Since the IEP of most proteins lies between pH 2 and 5, which, according to Drawert (1937), is also true of the proteins of the protoplasm and nucleus, for both dissociated fluorochrome groups the most favorable pH ranges for electroadsorptive storage are determined just by such model tests.

Of particular value in understanding the fluorochroming of smear preparations with different pH values is the test in a gelatin-benzene model carried out with the cathodic fluorochrome pyronine, a two-color fluorescence indicator. 5 cc of cooled but still liquid gelatin is mixed with 1 cc of benzene or toluene in a test tube and agitated. Every drop of this emulsion is spread out on slides and allowed to congeal.

On the slides are flat masses of gelatin containing microscopically small drops of benzene. These slides are then immersed for 15-60 minutes in pans with pyronine solutions at graduated pH values, after which they are washed in fluorochrome-free buffer solutions at the same pH values and examined under the FM. Table 10 shows the regularities observed in fluorochroming of such a model (Strugger, 1941).

In the extreme acid region and therefore below the IEP of the gelatin, which lies between pH 3.5 and 5, the gelatin is unable to adsorb the yellow fluorescing dye cations electrostatically since it is just as electropositively charged as the dye cations. If the IEP is exceeded, the gelatin is electronegatively charged and becomes intensely fluorochromed in electroadsorptive fashion by the yellow fluorescing pyronine cations. The more the dissociation of the pyronine decreases in the alkaline region, the weaker becomes the electroadsorptive staining of the gelatin by the hydrophilic pyronine cations. Finally a pH of 10 to 11 in the dye bath results in total inhibition of the dissociation and the gelatin does not take up any more of the yellow fluorescence color. On the other hand, the ultramarine blue fluorescing pyronine base, which is lipophilic, electively stains the drops of benzene through solubility affinity.

A protein-lipid system is therefore also maximally fluorochromable (through electroadsorption) by cathodic fluorochromes above the IEP in the maximum dissociation region of the fluorochrome. This fluorochroming, however, affects only the protein components. The lipid components, on the other hand, store the fluorochrome electively in the extreme alkaline region after dissociation ceases (Drawert, 1940).

Table 10

pH	(1) Benzol	Gelatine
2.03	---	+ gelh (2)
3.41	-	+ gelh
4.11	- + ultramarinblau (3)	+ + + + gelh
6.27	-- + ultramarinblau	+ + + + gelh
7.56	+ ultramarinblau	+ + + + gelh
11.21	+ + + + ultramarinblau	-

1 - benzene; 2 - yellow; 3 - ultramarine blue

pH Dependence of Fluorochroming of Smear Preparations with
Cathodic, Anodic, and Electroneutral Fluorochromes

My co-worker Denzler (1942) was the first to determine systematically the pH dependence of fluorochroming of fixed bacterial smears with cathodic, anodic, and electroneutral fluorochromes. The most significant results will be mentioned here. Bacterial smears of Sarcina lutea, Bacillus subtilis, and Bacillus mesentericus vulgatus as well as of Azotobacter chroococcum were fixed by heat or alcohol. The fixed smears were kept 5-10 minutes in buffered fluorochrome solutions, then washed 10 minutes in uncolored buffer of the same pH, and when dry examined under the FM. Since in practice it is often necessary for the fluorochroming of bacteria to have a suitable buffering of the dye solution, we have set down in Table 11 the buffer mixtures that proved to be effective in these investigations. The buffer solutions should be as fresh as possible.

Table 11

(1) Stammlösungen:

- n/10 HCl
I. 1/15 mol. primäres Kaliumphosphat (9,076 g in 1000 ccm Lösung, auffüllen im Maßkolben mit aqua dest.)
II. 1/15 mol. sekundäres Natriumphosphat 111,870 g in 1000 ccm Lösung.
III. 1/15 mol. tertiäres Kaliumphosphat 113,156 g in 1000 ccm Lösung

(2) Zusammensetzung:

(3)

Mengenangaben in ccm der Stammlösungen.

pH	n/10 HCl	I	II	III	$\frac{H_2O}{H_2O + Farbe(l)}$
2,0 - 2,2	9,5	0,5	—	—	$\frac{90}{90 + 10}$
3,4 - 3,9	0,5	9,5	—	—	$\frac{90}{90 + 10}$
4,0 - 4,9	—	10	—	—	$\frac{90}{90 + 10}$
5,6 - 5,7	—	9,5	0,5	—	$\frac{90}{90 + 10}$
5,8 - 6,1	—	9	1	—	$\frac{90}{90 + 10}$
6,1 - 6,5	—	8	2	—	$\frac{90}{90 + 10}$
7,0 - 7,1	—	6	4	—	$\frac{90}{90 + 10}$
7,5 - 7,6	—	2	8	—	$\frac{90}{90 + 10}$
8,0 - 8,3	—	4,5	—	5,5	$\frac{90}{90 + 10}$
9,8 - 10,1	—	6	—	4	$\frac{90}{90 + 10}$
10,7 - 10,8	—	3	—	7	$\frac{90}{90 + 10}$
11,2 - 11,3	—	—	3	7	$\frac{90}{90 + 10}$

(continued on next page)

(1) Standard solutions:

n/10 HCl

I. 1/15 mol. primary potassium phosphate (9.076 g in 1000 cc solution, fill up in MeB flasks with distilled water)

II. 1/15 mol. secondary sodium phosphate (11.870 g in 1000 cc solution)

III. 1/15 mol. tertiary potassium phosphate (14.156 g in 1000 cc solution)

(2) Composition:

(3) Quantities in cc of standard solutions

(4) Dye

The pH dependence of fluorochroming of Sarcina lutea smears with the cathodic auramine (standard "Bayer" dye) is shown in Table 12.

Table 12

pH	Fluoreszenz
2.72	(1) grünlich-gelb +
3.31	(2) grün-gelblich +++
6.34	(3) grün +++++
7.04	(3) grün +++++
11.13	(4) blau-grünlich +

1 - greenish yellow; 2 - green yellowish; 3 - green; 4 - blue-greenish

A very intense fluorochroming up to pH 9 does not appear in the protoplasm until after reaching the isoelectric zone of the bacterial proteins, which lies at about pH 3. At pH 11 auramine is no longer dissociated so that a greenish blue fluorochroming of the protoplasm lipids results from solubility affinity.

The results of treating Sarcina lutea and Bacillus mesentericus with the cathodic berberine sulfate are shown in Table 13.

Table 13

pH	<i>Haemillus mesentericus</i>	<i>Sarcina lutea</i>
2.15	(1) blaugrün-gelblich +	(2) fahl seegrün +
3.50	(3) hellgelblich-grün ++	(4) seegrün ++
6.50	(5) gelbgrün +++	(5) gelbgrün ++++
9.15	(6) gelblich-grün ++++	(5) gelbgrün ++++
11.42	(7) gelb-grünlich ++(+)	(5) gelbgrün ++(+)

1 - blue-green-yellowish; 2 - pale sea green; 3 - light yellowish-green; 4 - sea green; 5 - yellow-green; 6 - yellowish-green; 7 - yellow-greenish

Here too the pattern is the same. The maximum storage of berberine sulfate in the protoplasm was between pH 3 and 9. Below the IEP and consequently under pH 3 there was no significant fluorochroming. Of particular interest are the fluorochroming tests of *Sarcina lutea* smears with the cathodic pyronine, which is simultaneously a fluorochrome and a diachrome (Table 14, compare Tables 8 and 10).

Table 14

pH	(1) Fluoreszenzmikroskop	(2) Hellfeldmikroskop
2.24	(3) grün-gelblich +	keine Färbung (4)
3.32	(5) grünlich-gelb (+)	rotlich (6) +
6.94	(7) gelbgrün rötlich + gemischt	(8) rosa-karmesinrot ++++
9.34	(9) gelbgrün rötlich +	(8) rosa-karmesinrot +++(+)
11.21	(10) grünblau ++	keine Färbung (4)

1 - fluorescence microscope; 2 - bright-field microscope; 3 - green-yellowish; 4 - no dyeing; 5 - greenish-yellow; 6 - reddish; 7 - yellow-green reddish mixed; 8 - pink-crimson; 9 - yellow-green reddish; 10 - green-blue

The pyronine was so intensely stored above the isoelectric zone of the bacterial proteins that it was almost quenched as a fluorochrome, but as a diachrome it imparted a beautiful blue color to the bacteria. Since the pyronine is no longer dissociated at pH 11.2, no dye cation adsorption on the bacteria appears. Thus, no coloring can be determined in the bright field. When observed in the FM, however, the bacterial protoplasm now fluoresces gray blue and, therefore, in the color of the lipophilic pyronine base molecule. If brief dyeing with pyronine is carried out, fluorescence extinction recedes into the background in the maximum storage region and the protoplasm then fluoresces more intensely yellow when dyed at pH 3-5 to 9.

Anodic dyes behaved quite differently in fixed bacterial smears. The results of fluorochroming Sarcina lutea with fluorescein potassium are shown in Table 15.

Table 15

pH	(1) Hitzebehandlung	Alkoholisation
2.16	(2) gelb-grün + + +	(3) grün-gelblich + +
3.52	(3) grün-gelblich + + +	(2) gelb-grün + + +
6.44	(3) grün-gelblich (+)	(4) bläulich grün (+)
10.27	(5) blau-grün (+)	(5) blau-grün (+)
11.27	(5) blau-grün +	(5) blau-grün (+)

1 - heat fixation; 2 - yellow-green; 3 - green-yellowish; 4 - bluish-green; 5 - blue-green

Here the storage maximum lay in the extremely acid region and after the isoelectric point was passed the fluorochroming decreased irregularly. Denzler had the same results with the anodic primuline (Table 16).

Table 16

pH	(1) Hitzebehandlung	Alkoholisation
2.16	(2) blau-gelbgrün + + +	(3) blau-grün + + + +
3.52	(4) bläulich-grün + + + +	(5) blau-grün + + + +
6.44	(6) blau-grünlich +	(6) blau-grünlich + +
10.27	(6) blau-grünlich (+)	(6) blau-grünlich +
11.19	(6) blau-grünlich . (+)	(6) blau-grünlich +

1 - heat fixation; 2 - blue-yellow-green; 3 - blue-gray; 4 - bluish-green; 5 - blue-green; 6 - blue-greenish

The electroneutral rhodamine B showed no pronounced pH dependence of fluorochroming. The results of treating Sarcina lutea smears with the dye are shown in Table 17.

Table 17

pH	(1) Fluoreszenz
2.16	(2) mattgelb + (+)
3.52	(3) mattgelb-orange. + +
6.44	(4) grünlich-gelb + (+)
9.42	(5) gelblich + (+)
11.12	(6) goldgelb + +

1 - fluorescence; 2 - pale yellow; 3 - pale-yellow-orange; 4 - greenish-yellow; 5 - yellowish; 6 - golden yellow

Before writing this book, I reexamined Denzler's results by a comprehensive series of tests with numerous cathodic and anodic fluorochromes on heat-fixed smears of Bacterium coli, Sarcina lutea, Bacterium pyocyaneum, Staphylococcus aureus, Bacillus bombycis, and Spirillum. In all cases I was able in principle to achieve the same results as those presented in the above tables.

Thus, there can be no doubt that the isoelectric zone of fixed bacterial cell proteins lies between pH 2 and 4. Recently Nordmeyer (1947) used acridine orange to measure the IEP_M of some bacterial species. This method is based on the fact that below the IEP the bacterial proteins can store acridine orange only in a low concentration so that the bacteria fluoresce green, whereas the bacteria above the IEP can adsorb electrostatically the acridine-orange cations more intensely so that a copper-red fluorescence appears. Nordmeyer took their measurements in alcohol-fixed bacterial smears and obtained the following numerical values: Staphylococcus aureus IEP_M pH 2.81; Sarcina lutea IEP_M pH 3.46. The IEP of all the other bacteria_M investigated lies between these extreme values. In the acid region below the isoelectric zone occurs the maximum fluorochroming of the bacterial protoplasm for anodic fluorochromes, while the maximum for cathodic dyes above the isoelectric zone of the bacterial proteins lies between pH 4 and 8. The most favorable fluorochroming range for cathodic fluorochromes is at pH 5 to 7.

Theory

Analysis of the pH dependence of the fluorochroming of fixed bacterial smears with cathodic, anodic, and electroneutral fluorochromes has established a sound foundation for creation of a theory of fluorochroming. The patterns revealed are clear and readily reproducible. They can be briefly formulated as follows. There is a distinct relationship between the isoelectric zone of the bacterial proteins and the dye adsorption with cathodic and anodic fluorochromes in a dissociated state. The anodic fluorochromes color the fixed protoplasm in the acid region below the isoelectric zone, while the cathodic dyes fluorochrome maximally above the IEP in the weakly acid to neutral region. Moreover, there is a close relationship between the dissociation minimum of cathodic dyes lying in the more or less alkaline region and the fluorochroming. If the fluorochrome solution is presented in a pH range in which the dissociation has reached its minimum, no cation adsorption occurs, but only the more or less lipophilic color base fluorochromes the structural lipids of the fixed protoplasm. In accordance with these regularities, there can be no doubt that the anodic and cathodic fluorochromes color the protoplasmic body of the bacteria in electroadsorptive fashion. The analogies to the results of the model tests are so clear that there is no need of more detailed discussion of these relations. The electroneutral fluorochromes and the molecular color base solutions of cathodic fluorochromes fluorochrome the structural lipid complex of the protoplasts through solubility affinity. Hence, no pronounced pH dependence could be found for the electroneutral fluorochromes.

The Practice of Fluorochroming Fixed Bacterial Smears

On the basis of theoretical studies we can immediately state which of the three groups of fluorochromes is best suited for fluorescence-optical preparation of bacterial smears. Since the protein and membrane components constitute the chief fluorochromable part of the bacterial cell and the structural lipids represent only a slight percentage of the bacterial body, electroneutral fluorochromes and the molecular color base solutions of cathodic fluorochromes cannot be used in the extreme alkaline region because of insufficiently intense fluorescence. The fluorochroming of the lipid components is only of theoretical interest because it shows us that the bacterial protoplasm too abounds in structural lipids and that it does not differ in fundamental structure from the protoplasm of other protophytic and metaphytic cells.

Fluorochroming with cathodic and for special purposes also with anodic fluorochromes, on the other hand, is extremely significant. The cathodic fluorochromes in particular are well suited for smear fluorochroming because of their characteristics. Their cations generally fluoresce intensely in aqueous solution. The intensity of fluorescence increases quite markedly with most cathodic fluorochromes when in an adsorbed state. The pH range in which they yield the best results is likewise favorable and it is checked simply by dissolving the fluorochrome in distilled water. Certain anodic fluorochromes are excellent for special purposes, e.g., for elective spore dyeing. However, they are less suitable for the general fluorochroming of bacterial smears because the fluorochroming optimum is reached only in an extremely acid solution.

A General Method of Fluorochroming Bacterial Smears

The purpose of a general method of fluorochroming is to make any bacterial smear accessible to FM observation by means of fluorochroming. General fluorochroming, therefore, parallels the usual smear dyeing of bacterial preparations with diachromes.

Table 18 lists a number of cathodic fluorochromes already tested. The fluorescence colors are also noted.

Table 18

Name	(11) Fluoreszenzfarbe in wässriger Lösung	(12) Bemerkungen
Acridinorange	(3) laubgrün	(7) in stärker adsorbiertem Zustand rot
Acridingelb (1)	(3) laubgrün	
Acridinrot (2)	(4) kreß	
Auramin	(5) Spur	(8) in adsorbiertem Zustand stark gelblich
Auramin G	(3) laubgrün	
Deazauramin	(3) laubgrün	
Debernasulfat	(5) Spur	(9) in adsorbiertem Zustand laubgrün
Carbocyanin G	(3) laubgrün	
Carbocyanin	(3) laubgrün	
3,6-Diaminocarbocyanin	(3) laubgrün	
Fuchsin WMX	(3) laubgrün	
Methylorange ND	(3) laubgrün	
Kisanil	(3) laubgrün	
Phosphin 2 H	(3) laubgrün	
Phosphin N	(6) gelb	
Pyronin	(6) gelb	(10) in stärkerer Konzentration gelbsch, Eigenfarbe dann rot

1 - acridine yellow; 2 - acridine red; 3 - leaf green; 4 - cress;
 5 - trace; 6 - yellow; 7 - red in more intensely adsorbed state;
 8 - intense golden yellow in adsorbed state; 9 - leaf green in adsorbed state;
 10 - quenched in stronger concentration, intrinsic color red;
 11 - fluorescence color in aqueous solution; 12 - remarks

Instructions for fluoro-chroming:

The bacterial material spread on a slide is heat-fixed in the usual way, covered with a 1:100-1:1000 fluoro-chrome solution in distilled water, and dyed in the cold for 10-60 seconds. It is then rinsed with distilled water. After the rinsed smear dries, the preparation is completed. The following is to be noted in connection with the time required. There are substances which fluoro-chrome so quickly that over-dyeing can occur, thereby damaging the intensity of fluorescence of the bacteria. In such cases the duration of fluoro-chroming should be kept as short as possible. Other fluoro-chromes

require more time but still yield good results even after a short period of time. If a preparation is to be observed with a cover glass, the maximally pure nonfluorescing liquid paraffin rather than the fluorescing Canada balsam is used as the embedding medium. The liquid paraffin is placed on the dried smear.

As the microphotographs (Plate I, 2, 3, 4) show, the resultant pictures are impressive. Against the completely black background the bacteria appear to the observer as independent colored light sources. They stand out in shape and, with careful fluorochroming, also in content so that it can be rightly said that fluorochromed bacterial smear preparations facilitate both the detection of individual bacterial forms and the study of details as compared with the diachroming method. Another important factor from the standpoint of laboratory practice is the extraordinary amount of time saved in producing the preparation, due mostly to the brief period of dyeing required. The preparation can be observed with any improvised blue-light or UV-light FM. Thus, special and expensive apparatus is not necessary.

The preparation of Micrococcus gonorrhoeae in heat-fixed pus smears with the cathodic fluorochrome coriphosphine was impressive. If not used in excess, coriphosphine produces green fluorescence in the cytoplasm of leukocytes and copper-red fluorescence in the nuclei. The extracellular and intracellular gonococci acquire a bright copper-red fluorescence. These color relationships make it possible to observe the extracellular gonococci in copper-red fluorescence against a black background and the intracellular gonococci in the protoplasm of the leukocytes in copper-red fluorescence against a green background. The procedure is as follows:

- (1) Heat fixation of the pus smear.
- (2) 1-2 minutes' dyeing of the smear at a normal temperature with a 1:100 coriphosphine solution in distilled water poured over it.
- (3) Rinsing of the smear with distilled water.
- (4) Drying of the smear in the air.
- (5) Covering with liquid paraffin under a cover glass. The smear can also be observed without a cover glass in a dry state with fluorescence-free immersion oil.

This coloring of gonococci is, of course, not specific, but it should make it much easier to detect the microorganisms especially in leukocytes. We must emphasize the great saving of time in producing the preparation. Such a preparation can be observed within 2-3 minutes.

Haitinger and Schwertner (1939) mentioned fluorochroming with special acridine yellow in preparing gonococci. Pus smears were kept for 5 minutes in a special 1:500 acridine yellow solution in distilled water and then rinsed with distilled water. The nuclei of the pus cells shone green-yellow, the protoplasm dark green, and the gonococci intense golden yellow.

The anodic fluorochromes that have been found to be of practical use in my laboratory are listed in Table 19 along with their fluorescence colors.

Table 19

Name	(1) Fluoreszenzfarbe in wässriger Lösung
3-Aminopentrisulfonsäures Natrium	(3) laubgrün
Brillantsulfobäurin FF	(3) laubgrün
1,3-Dihydroxynaphthalen-5,7-disulfonsäure	(4) seegrün
Eosin	(3) laubgrün
(2) Kaliumfluorescein	(3) laubgrün
Oxypentrisulfonsäures Natrium	(5) laubgrün-seegrün
Sulfocandurin G	(6) gelb

1 - fluorescence color in aqueous solution; 2 - potassium fluorescein; 3 - leaf green; 4 - sea green; 5 - leaf green-sea green; 6 - yellow

The anodic fluorochromes can be successfully used only in acid solution. The procedure is as follows:

- (1) Heat fixation of the bacterial preparation.
- (2) Dyeing of the smear with fluorochrome solutions to which is added 0.5 cc of n/1 HCl in 100 cc of aqueous solution in distilled water (1:1000). Dyeing takes from 10 seconds to 5 minutes. The dyeing time for each fluorochrome must be determined empirically.
- (3) Brief and vigorous rinsing with distilled water.
- (4) Air drying.

Fluorochroming of Bacterial Spores

The dyeing of bacterial spores is carried out on heat-fixed spore-containing smears with carbolfuchsin-methylene blue. The spores are colored red while the remaining bacterial body or nonsporulating bacillus appears to be colored blue. The entire procedure requires at least 15 minutes. Pesch (1959) reports for the first time on a procedure for FM identification of bacterial spores. Fixed smears are treated either with hydrogen peroxide or preferably with 5% chromic acid (5-10 minutes). They are then dyed with a fluorochrome like auramine. For elective dyeing it is necessary to wash the fluorochrome out of the nonsporulating bacteria or to quench it. This is done with some potassium permanganate solution. The bacterial cells can then barely be seen in the FM while the spores electively fluoresce green-yellow. Clauberg (1959) reports the same method.

In the interest of saving time and making identification easier, it is worthwhile to examine the problem of spore fluorochroming more closely. I worked out the following principles after studying countless fluorochromes with respect to their ability to dye sporulating and nonsporulating bacterial forms (Strugger, 1946). In general, the spores stained very poorly with the cathodic fluorochromes. With careful observation they can be recognized, as a rule, in an otherwise well fluorochromed bacterium as empty space without special fluorescence. Only in the group of anodic fluorochromes could I find some that in acid solution clearly fluorochromes the spores either in their membrane or in toto. These fluorochromes included especially brilliant sulfoflavin FF owing to the intensity of the fluorescence produced. This fluorochrome is really quenched and distinguished for its unusual diffusibility in gelatin. Its particle size, therefore, is very small. Hence it can quickly and easily penetrate into the membrane and interior of the fixed spores and excite intense secondary fluorescence. In comparison with other anodic fluorochromes, I gained the impression that it is mainly the finely dispersed condition of the solution that enables this dye to fluorochrome spores.

Besides the spores, all the nonsporulating bacteria produced an intense green-yellow fluorescence. Although the spores have an elective and particularly intense secondary fluorescence, the simultaneous dyeing of the bacillus signifies some impairment of the desired differentiation of these colors.

On this basis I worked out with the help of a coarsely dispersed cathodic fluorochrome that fluoresces in another color contrast dyeing of the nonsporulating bacteria and surviving plasmatic bodies of sporulating bacteria. Coriphosphine (also flavophosphine) is best suited for this contrast fluorochroming because it is so coarsely dispersed that it cannot penetrate quickly into the membrane or interior of the spores, although it produces copper-red fluorescence in the nonsporulating

bacterial protoplasts and the protoplasmic regions lying outside the spores. The brilliant sulfoflavin-coriphosphine fluorochroming of spore-containing preparations shows the following picture in the FM. All the spores are fluorochromed bright green-yellow by the brilliant sulfoflavin. The protoplasm lying outside the spores and the nonsporulating bacteria shine copper red. If nonsporulating bacteria are not so treated, all the bacterial cells appear copper red (Plate I, 5).

The theory underlying this spore staining technique is more or less clear. The anodic dye brilliant sulfoflavin FF colors the spores in strong acid solution because of their ready permeability. Similarly, it fluorochromes all the remaining protoplasmic regions and the nonsporulating protoplasts into green-yellow fluorescence. In the case of contrast fluorochroming with a more or less neutral aqueous coriphosphine solution (cathodic fluorochrome), superposition of the brilliant sulfoflavin fluorochroming of the spores is no longer possible owing to the coarser dispersity of the coriphosphine, although the dye cations of the copper-red fluorescing coriphosphine penetrate into the remaining protoplasm and into the nonsporulating bacterial bodies. This is overlain in electroadsorptive fashion with the opposite charged coriphosphine so that a copper-red secondary fluorescence appears. The desired differentiation is thus achieved.

The technique of brilliant sulfoflavin-coriphosphine fluorochroming includes the following:

- (1) Good heat fixation of the smear.
- (2) Covering the slide with a brilliant sulfoflavin solution (1:400) in distilled water in which 0.5% phenolum liquefactum is dissolved by vigorous shaking. Gentle heating of the slide over a flame until the formation of vapor. Duration of dyeing 1-2 minutes (somewhat longer with many types of spores).
- (3) Good rinsing with distilled water (wash bottle).
- (4) Redyeing with a drop of a coriphosphine solution (1:1000) in distilled water. Duration about 40 seconds.
- (5) Rinsing with distilled water.
- (6) Drying of the smear.

The investigation can be carried out with any improvised FM. If a cover-glass preparation is to be studied, it is embedded in liquid paraffin.

Such preparations exhibit a beautiful contrast effect and can be kept in the dark for quite some time. The behavior of different bacterial spores in the presence of brilliant sulfoflavin is variable. For example, the spore material of Bacillus mycoides generally shows thorough dyeing of the total spore contents. Bacillus bombycis and

Bacillus mesentericus spores, on the other hand, reveal a very bright fluorochroming of the solid spore membrane. Parallel dye tests with the familiar carbolfuchsin dye produced the same differences. It must be emphasized, furthermore, that unfixed bacterial spores do not produce unequivocal results with brilliant sulfoflavin and that the failure to add phenol has unfavorable consequences.

The recently developed spore fluorochroming technique is a clear optical method of detecting the presence of bacterial spores in a smear. One of its advantages is that the presence of even small quantities of spores can be easily detected with rather weak magnification. Moreover, brilliant sulfoflavin fluorochroming requires only 3 minutes, a fifth of the time previously required for color detection of bacterial spores.

May (1947) recently published a short report on simple contrast dyeing of spores with sodium oxypyrene trisulfate (cf. Strugger, 1939, for more details on the use of this dye). This intensely fluorescing acid fluorochrome dyes bacteria a green fluorescence color in alkaline solution and a blue color in acid solution. If sporulating bacteria are dyed in acid sodium oxypyrene trisulfate and in acridine orange solution, the bacilli fluoresce red-brown and the spores blue. As compared with brilliant sulfoflavin, my personal experience shows that sodium oxypyrene trisulfate does not produce as strong a fluorescence contrast. The procedure is as follows. 50 cc of a 1:500 sodium oxypyrene trisulfate solution in water is mixed with 50 cc of $n/2$ HCl and 2 cc of a 1:1000 acridine orange solution in water. The air-dried or heat-fixed smear is covered with the dye solution, dyed for 5 minutes, and then dried. The preparation must be examined with an UV-light FM since blue fluorescence cannot be seen in a blue-light FM.

Elective Fluorochroming of Mycobacterium tuberculosis and Other Acid-Fast Types

In 1937 Hagemann laid the foundation for the fluorochroming of fixed bacterial smear preparations by developing the FM method of detecting leprosy bacilli in smears of nasal mucus and blood. This work was immediately noticed because it clearly demonstrated the advantages of fluorescence microscopy for bacteriological research. According to Hagemann, the acid-fast leprosy bacilli can be fluorochromed with berberine sulfate in distilled water (1:1000) with the addition of 5% phenolum liquefactum. The smears are dyed for 15 minutes at a normal temperature. Then hot water (60-70°) is used for differentiation. The best procedure is to bathe the preparation in a heavy stream of the water. This decolorization process takes 10-50 seconds. The nonacid-fast bacteria are decolorized in this way. Only the leprosy bacilli stand out in intense yellow to green colors against the very weakly fluorescing background.

In 1938 Hagemann discovered a new and more suitable fluorochrome for FM detection of leprosy and tuberculosis bacilli in auramine. Standard "Bayer" auramine imparts a shiny golden yellow fluorescence to the bacteria. After differentiation with HCl-alcohol, only the acid-fast types remain fluorescent while the nonacid-fast accompanying bacteria become decolorized. Dyeing instructions call for the following:

- (1) Heat fixation of the smear.
- (2) 15 minutes' decolorization at a normal temperature in auramine solution(1:1000)(distilled water) plus 5% phenolum liquefactum (shaken thoroughly).
- (3) Vigorous rinsing with tap water.
- (4) Differentiation with HCl-fuel alcohol (1000 cc of alcohol + 4 cc of pure HCl DAB + 4 g of NaCl) for 3 minutes, with the HCl-alcohol necessarily replenished after 1½ minutes.
- (5) Repeated vigorous rinsing with tap water.
- (6) Air drying of the preparations.

The preparations are examined either in a dry state without cover glasses with dry lenses (for more precise work, with an immersion system) or they can be placed under cover glasses with maximally pure and barely fluorescing liquid paraffin. On gross inspection the preparations look to be unstained. The nonacid-fast nuclei are no longer visible under the microscope because of decolorization by HCl-alcohol. Leprosy or tuberculosis can be diagnosed if a preparation has the golden yellow fluorescing bacilli with the form characteristic of the tubercle bacilli. The acid-fastness of the tubercle bacilli is not always constant. Bacilli with reduced acid-fastness sporadically exhibit only a weak greenish or pale gray fluorescence.

Hageman's auramine fluorochroming technique was improved by Herrmann in 1938. It involves:

- (1) Heat fixation of the smears.
- (2) Dyeing with Hagemann's (1938) auramine-phenol solution and simultaneous heating over a Bunsen burner to a boil. Duration 5-6 minutes.
- (3) Rinsing with running water.
- (4) Differentiation with 70% fuel alcohol containing 3% HCl until the preparation is colorless. Duration 15-20 seconds.
- (5) Rinsing with running water.

- (6) Immersion in a 1:1000 potassium permanganate solution for exactly 5 seconds.
- (7) Rinsing with running water.
- (8) Immersion in Löffler's alkaline methylene blue for 1 second.
- (9) Rinsing and drying.

This fluorochroming procedure for acid-fast microorganisms has the advantage that all the fluorescence phenomena are quenched in other parts of the preparation except in the tubercle bacilli so that the differentiation becomes even more clear-cut. Rihl (1940) was able to confirm the intensification of the contrast produced by Herrmann's method.

Keller (1938) tested the usefulness of the auramine method and had the merit of introducing blue-light fluorescence microscopy for observation of auramine fluorescence of tubercle bacilli. Since auramine in an adsorbed state shows a bright golden yellow fluorescence, there is absolutely no need to use a UV FM with quartz lenses to carry out tuberculosis detection. Auramine fluorescence can be excited just as well if not better by blue light and a suitable absorption filter ensures contrast. Keller proposed the use of a low-voltage microscope lamp as a light source for tubercle bacillus detection. It was recognized by Keller as being useful for this special case. It is doubtful whether its light intensity is sufficient for all cases and for precise FM tuberculosis diagnosis it is perhaps better to call upon a stronger blue-light source (mercury-vapor lamp, carbon-arc lamp with suitable filters).

The advantages of Hagemann's auramine fluorochroming of tubercle bacilli over the Ziehl-Neelsen method are obvious. The luminescence of the tubercle bacilli against a dark background makes it much easier to find isolated bacilli. Plate II, 1 shows a microphotograph of tubercle bacilli fluorochromed with auramine by Hagemann's method. Another important consideration in the practical management of a research laboratory is the saving of time gained by using the FM method. Judging by experience to date, it is more than double.

Tests of fluorochroming of tubercle bacilli have also had favorable results in the practical operation of clinics and research laboratories. Herrmann (1938) clearly demonstrated by 1424 tests the superiority of the fluorescence method to Ziehl-Neelsen dyeing. Clauberg (1939) critically examined the usefulness of the auramine procedure and in about 500 tests found 18% more positives than in Ziehl-Neelsen smears. In no case did the Ziehl-Neelsen preparation yield a positive finding with the fluorescence preparation yielding a negative one at the same time. Unfortunately, the removal of other acid-fast bacteria is technically impossible. Therefore, in fluorescence microscopic diagnoses

the same reservations must be made as in the case of Ziehl-Neelsen preparations. Clauber, says: "All in all we have to conclude that fluorescence-microscopic tubercle bacillus detection must be acknowledged the method of choice owing to the greater technical ease of handling and more positive yields as compared with the procedures used up to now." Didion (1939) compared the efficiency of the fluorescence and Ziehl-Neelsen methods on 702 samples of tuberculosis-suspect material. If there were suspicious bacilli in the FM preparation alone with a simultaneously negative finding in the Ziehl-Neelsen preparation, an animal test was carried out. There was a total of 147 positives among 573 sputums. Of these, 129 were positive with both methods, 0 with the Ziehl-Neelsen alone, 8 with the FM method alone, and 10 with cultures alone. The animal test was positive in the 8 cases established by fluorescence. Didion ranked the efficiency of the fluorescence procedure as follows: animal test 100, cultivation test 92, fluorescence microscopy 87.2, and Ziehl-Neelsen method 82.2. A detailed quantitative comparison of the Ziehl-Neelsen and auramine preparations showed that the same number of tubercle bacteria was dyed in both and that, consequently, the two methods make possible a thorough quantitative dyeing of tubercle bacilli. However, the superiority of the FM method with respect to ease of observation is beyond question.

Dabelstein (1939) on the basis of abundant research material reaches the same positive conclusion. Jung (1940) compares fluorescence diagnosis with tests on guinea pigs. Animal tests are undoubtedly superior to the fluorescence method, but Jung was able to satisfactorily reconcile the two. Of 225 sputums investigated, two proved positive with the fluorescence method while one was negative in an animal test. This is not to be wondered at, for despite the high resistance of tubercle bacteria, a small number of dead bacilli sometimes appears in sputum. Complete substitution of fluorescence microscopy for animal experiments is perhaps impossible.

Haitinger and Schwertner (1939) made a successful attempt to develop a new technique for dyeing tubercle bacilli. They criticized the laboriousness and complicated procedures involved in Hagemann's auramine method and Herrmann's modification thereof. The fluorochrome acridine yellow extra dyes tubercle bacteria in sputum smears in 20-30 seconds at the most so that the same effect is achieved with a considerable shortening of the fluorochroming time. For acridine yellow extra-fluorochroming the two authors suggest the following procedure:

- (1) Heat fixation of the preparations.
- (2) Pouring on 1:5000 acridine yellow extra-solution in distilled water and rinsing the preparation with water after 30 seconds.
- (3) Differentiation with 4% HCl-alcohol for about 10-15 seconds until the preparation seems colorless in daylight.
- (4) Rinsing with water and drying.

The entire procedure can therefore be carried out in about three-quarters of a minute.

Should the method recommended by Haittinger and Schwertner prove in practice to be just as good as auramine fluorochroming, it would represent a major technical advance.

Bachmann and Finke (1939) successfully attempted to apply Hagemann's auramine method to tissue sections. Paraffin sections are handled as follows:

- (1) Removal of paraffin with xylene for 3 minutes.
- (2) Passage through decreasing alcohol series.
- (3) Vigorous rinsing with water.
- (4) Pouring on 1:1000 auramine solution containing 5% phenolium liquefactum. Cold dyeing takes 15 minutes.
- (5) Rinsing with water.
- (6) Differentiation with fuel alcohol to which 4% HCl and 4% NaCl have been added, 3 minutes. The solution is replenished after 1½ minutes.
- (7) Rinsing with water.
- (8) Covering with 1:10 aqueous methylene blue solution for about 30 seconds.
- (9) Rinsing with water and careful drying between filter paper.

Embedding of cover-glass preparations in nonfluorescing immersion oil is suggested.

The tissue structures are still largely recognizable and the bright yellow fluorescent tubercle bacilli can be localized best. Also scattered tubercle bacilli are easy to see.

Schallock (1940) uses thiazole yellow for contrasting tissue sections fluorochromed with auramine. The golden yellow fluorescing tubercle bacilli stand out against the weakly blue fluorescing tissue, thus helping to heighten the contrast. The technique is as follows:

- (1) Removal of paraffin.
- (2) Descending alcohol series.
- (3) Rinsing in water.

- (4) 5 minutes' dyeing with auramine (1:1000) at 37°.
- (5) Decolorization in HCl-alcohol for 1-3 minutes at 37°.
- (6) Rinsing with distilled water.
- (7) 2 minutes' dyeing with thiazole yellow (1:1000).
- (8) Rinsing in 50% alcohol.
- (9) Rinsing with water.

The material should not have been fixed longer than 4-5 days in formalin.

In summary, the FM method of tubercle bacillus detection is now officially regarded as an extremely useful diagnostic method. There is no doubt that it can be improved still further and thus lighten the load of bacteriologic research laboratories.

Elective Fluorochroming of Trypanosomes in Blood Smears*

Trypanosomes can be so clearly fluorochromed in fixed blood smears that the resultant pictures are far superior to those obtained after Giemsa staining. After this treatment the red blood cells fluoresce a dull dark green while the trypanosomes fluoresce a bright golden yellow. They can then be easily found on examination of the preparation with a weak objective as long as they are isolated. This undoubtedly facilitates the identification of trypanosomes. The fluorochroming procedure involves the following steps:

- (1) Preparation of the blood smear.
- (2) Fixation of the air-dried smear in concentrated methyl alcohol for 3 minutes.
- (3) Rinsing with distilled water.
- (4) 4 minutes' dyeing with 1:1000 auramine in distilled water plus 5% phenolum liquefactum.
- (5) Rinsing with distilled water.
- (6) Drying and examination.

* This procedure was worked out jointly with Dr. Ullmann of Trendelburg on the occasion of a visit to the Botanical Institute of the Hannover Veterinary College due to practical necessity.

The examination itself is best carried out with a blue-light FM. The golden yellow fluorescence of the trypanosomes is so intense that even improvised blue-light devices with low-voltage or arc lamps can be used as light sources.

Fluorochroming of Virus Particles

According to Hagemann (1937), filterable canary, mousepox, and smallpox vaccine viruses can be easily fluorochromed with primuline without special differentiation. The procedure is as follows. 1 g of primuline is dissolved in 1000 cc of distilled water, 20 cc of phenol liquefactum added, and the mixture vigorously agitated. The solution can be kept several days in the dark. The unfixed smears are covered with the primuline solution and dyed for 15 seconds. They are then rinsed with distilled water and dried.

The virus particles appear against a dark background as white to white-bluish fluorescing spots. An immersion objective is used for observation.

Clauberg (1939) became convinced of the usefulness of Hagemann's primuline method after experiments on smallpox virus. Gerlach's data (1937/1938) on elementary particles in smears from human and animal carcinomas and sarcomas, however, could not be confirmed by Clauberg. Haitinger (1938) published a survey of the present status of virus fluorescence microscopy.

FLUOROCHROMING OF LIVING MICROORGANISMS (ACRIDINE ORANGE METHOD)

Introduction

To stain living protoplasts without seriously disturbing the vital function of the cell and thus permit convenient observation of all the cytological units *intra vitam* has been a goal of biomedical research pursued vigorously since the introduction of vital staining technique by Pfeffer in 1886. Until recently numerous investigators worked on the problem but were unable to solve it. It was therefore widely believed that the protein ground substance of living protoplasts could not be stained *intra vitam* and that only dead, fixed protoplasmatic protein could be so strongly stained as to show up in the microscope.

Most of the investigators sought to achieve vital staining of protoplasm with diachromes. The basic (cathodic) diachromes produced, however, only distinct staining of vacuoles and granules but not of the ground substance. Likewise the nuclei are clearly, *supravital*ly stainable with basic dyes only in a pathologically altered state. "Vital staining of protoplasm" (Kuster, 1926; Albach, 1927, 1928; Gicklhorn, 1914, 1927) was, to be sure, achieved with acid dyes like eosin and erythrosin, but Strugger's analysis (1931) showed that such cells incur extensive premortal injury.

The reason why vital dyeing of living protoplasts with diachromes is technically impossible can now be understood. Intraplasmatic dye concentrations must be quite high for a diachrome to produce a useful effect in the microscopically thin layer of a preparation and in the still thinner layer of the cell structures. Comparative experiments with intensely staining dyes of different concentrations involving cover-glass preparations empty of all but such dye solutions show that really useful observation of the dye effect in transmitted light is impossible until dye solutions in concentrations of 1:100 to 1:1000 are used. Therefore, 1:100 to 1:1000 concentrations can be correctly assumed as the useful minimum of intraplasmatic concentration. The adherence of an alien substance in such a high concentration to the plasmatic proteins must naturally have unfavorable consequences for the protoplasts. It is easy to understand, therefore, why all the authors report that diachromes injure the protoplasts.

Solution of the problem of vital staining of protoplasts with diachromes is thus impossible from the theoretical standpoint and, as results to date show, from the practical standpoint as well. The limits of optic identification sensitivity in a microscopically thin layer are too unfavorable for diachromes.

The situation is quite different, however, when fluorochromes are used to solve the problem. Some time ago Metzner (1919, 1924) was able to observe vital fluorochroming of protoplasm in protozoans with numerous fluorochromes in conjunction with a study of the photodynamic phenomena. The studies of Ellinger and Hirt (1930) on intravital microscopy in luminescence light led to introduction of fluorochroming with acriflavine into research on physiological processes in animals. Schumacher (1933, 1936) using anodic potassium fluorescein succeeded in achieving the vital fluorochroming of the protoplasts of plant cells without seriously disturbing the protoplasm or motility. He used this observation to investigate diffusion of substances in plant cells. Doring (1935) studied potassium fluorescein storage in higher plant cells in great detail and found that only the living protoplasm through treatment with potassium fluorescein takes on an intense green secondary fluorescence in its ground substance, whereas the dead protoplasm does not fluoresce at all.

Potassium fluorescein staining was thoroughly analyzed by Strugger (1938). In 1940 Bukatsch and Haitinger and Strugger discovered at the same time and independently of each other the use of acridine orange as a fluorochrome for vital staining. Bukatsch and Haitinger mention in their work (1940) that the protoplasm of living plant cells fluoresce after being treated with acridine orange. Strugger (1940) made for the first time a comprehensive physical-chemical and cell-physiological analysis of acridine orange fluorochroming and discovered the following important facts:

1. Acridine orange has a pronounced affinity for the living proteins of protoplasts. Therefore, an extremely rapid and sparing vital staining of all important cytological structures with this fluorochrome is possible.

2. Fluorochroming, if carried out carefully, is harmless and the cells continue to function normally.

3. Living and dead protoplasm can be fluorochromed in different fluorescence colors with all desirable exactness. Living protoplasm can be fluorochromed only green, dead protoplasm only copper-red.

4. The cause of this phenomenon was revealed by the discovery of the concentration effect.

Bukatsch (1941) checked this finding of Strugger and confirmed it in all details. Höfler (1947) and Kölbel (1947) likewise provided full confirmation of the above facts.

Strugger (1940) observed that vital fluorochroming of chromosomes with acridine orange in dividing cells did not disturb the course of mitosis.

In 1940 Strugger was the first to culture a protophyte in a cytologically completely fluorochromed state. Didymium nigripes myxamoebas whose cytoplasm and nuclei were fluorochromed vital green with acridine orange continued their normal development. Copulation, plasmodium formation, sporophore development, and sporulation proceeded in normal fashion, even though all the living protoplasm was fluorochromed with the desirable clarity.

In 1941 Strugger was the first to use the acridine orange method to distinguish between living and dead yeast cells. These observations were analyzed on a broader basis in 1943. It was shown that living and dead yeast cells can be clearly and quickly differentiated by this method. Suitable isolation and cultivation experiments revealed that yeast cells whose cytoplasm and nuclei were distinctly fluorochromed green could continue to develop normally.

In 1942 the acridine orange method was extended by Strugger and Hilbrich to the vital staining of bacteria. FM differentiation of living and dead bacterial cells became possible and the cultivation of vitally fluorochromed bacteria was carried out in conjunction with animal experiments.

In 1944 Strugger and Rosenberger fluorochromed the spermatazoa of the goat vitally in all essential parts (head and tail) with acridine orange. Artificial insemination experiments with vitally stained spermatazoa were completely successful.

Johannes (1945) observed the fungus Phycomyces blakesleeenanus grow in a vitally fluorochromed state. All the protoplasm and the entire nuclear apparatus of this fungus were thoroughly fluorochromed in normally growing individuals.

Asher (1947) carried out total cytological vital fluorochroming in vertebrates (frogs and mice). An injection of acridine orange either subcutaneously or into the lymph sac quickly produced a fluorochroming of all the living protoplasts in the animal (blood, muscle cells, organ cells, nerve cells, sex cells). The nuclei were completely stained. The animals continued to live normally. Thus, acridine orange can be used for intravital fluorochroming of all the cells in an animal. This should be a highly significant discovery for the further development of cytological, histological, and physiological problems.

The foregoing data show beyond doubt that the problem of intravital staining of protoplasm can be solved by using fluorochromes. The reasons for achieving vital fluorochroming are theoretically easy to understand. The optical identification sensitivity for fluorochromes in the FM is much greater than that for diachromes in the bright-field microscope. Consequently, to obtain a clear localizable fluorochroming, it is not necessary to increase the intraplasmatic concentration as much as in vital staining of cells with diachromes. The fluorescence optical, readily detectable intraplasmatic concentration lies between 1:1000 and 1:10,000. Use of the most suitable, nontoxic fluorochrome, therefore, puts the problem of the vital stainability of the protein components of living protoplasm by the fluorescence method within the range of possibility both theoretical and practical.

In view of the broad prospects presented by acridine orange fluorochroming for biology and medicine, it is not inappropriate to accord a relatively large amount of space in this book to summarize the experience gained to date in the field of microbiology.

Microbiology has acquired a new dye technique based on cell-physiological principles and it cannot be directly compared with the earlier methods used for microorganisms. The acridine-orange technique is a physiological method which in its program goes far beyond the previously existing concepts of histological and bacteriological staining techniques.

Investigation of vital and lethal fluorochroming of protoplasm constitutes, moreover, the starting point for a new development of methods for the indirect study of the submicroscopic structures of protoplasm.

Chemistry and Characteristics of Acridine Orange

The fundamental substance of the acridine dyes, which include the medically important acriflavine, atebrin, and rivanol, is acridine (Fig. 9).

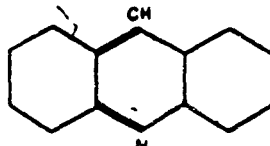


Fig. 9. Acridine.

The dye acridine orange is made not from this fundamental substance but from synthesizing it from asymmetrical dimethyl-m-phenylenediamine. This forms with formaldehyde the hydroacridine derivative which is oxidized to 3,6-tetramethyldiaminoacridine by atmospheric oxygen. The structural formula of acridine orange is shown in Fig. 10. It is used commercially to dye tanned cotton.

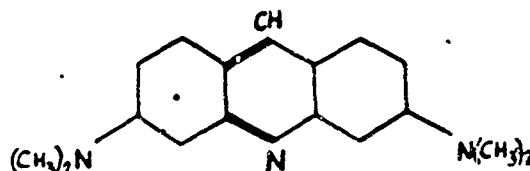


Fig. 10. Acridine orange, 3,6-tetramethyldiaminoacridine.

The basic (cathodic) nature of the dye is clearly evident from the structural formula. In the dissociated state the fluorescing part is represented as cation. The molecular color base must be strongly lipophilic owing to the end methyl groups, while the color cation must possess purely hydrophilic properties. If a 1:1000 acridine orange solution is prepared in distilled water and a drop of the solution is placed on filter paper, this capillary-analytical fundamental experiment confirms the basic (cathodic) nature of the dye. The coloring part of the dye solution is tenaciously held on the surface of the filter paper fibers by electroadsorption while the solvent water forms a broad colorless ring that spreads quickly by capillary action. Acridine orange is both a diachrome and a fluorochrome.

Characteristics as a Diachrome

Acridine orange has an inherent orange to brown-yellow color, but it can hardly be considered for microscopic staining because the color intensity is relatively weak in a microscopically thin layer. Like all diachromes, the characteristics of acridine orange too change with the concentration. Table 20 shows the intrinsic colors of 1.5 cm layers

of solutions of this dye in distilled water according to Ostwald's color scale (produced in transmitted light).

Table 20

(1) Konzentration	(2) Eigenfarbe
1:100 000	(3) gelb 2 ea
1: 50 000	gelb 2 ga
1: 10 000	gelb 2 pa
1: 5 000	gelb 3 aa
1: 1 000	(4) kreß 4 pa
1: 500	kreß 5 pa
1: 100	kreß 6 aa

1 - concentration; 2 - intrinsic color; 3 - yellow; 4 - cress

It is evident that in the dilute solution region acridine orange is colored bright yellow in transmitted light. The yellow color deepens with increasing concentration and it gradually changes into cress at 1:1000, finally becoming a very dark cress until 1:100.

In order to determine how dependent the intrinsic color of acridine orange is on the hydrogen ion concentration, 1:10,000 acridine orange solutions were buffered with phosphates in such a way that 10 solutions with increasing pH were obtained (Table 21).

Table 21

pH	(1) Eigenfarbe
2.37	(2) gelb 2 pa
2.25	gelb 2 pa
4.07	gelb 2 pa
5.88	gelb 2 pa
6.97	gelb 2 pa
7.08	gelb 2 pa
7.90	gelb 2 pa
9.15	gelb 3 aa
10.96	gelb 1 aa

1 - intrinsic color; 2 - yellow

It is evident that the yellow tone of the acridine orange solution does not change from the extreme acid region until beyond the neutral point, but over pH 9 a distinct change into a bright, somewhat greenish yellow takes place.

Thus, acridine orange as a diachrome has weak indicator properties and the indicator transition zone lies between pH 8 and 9.

If the color bases obtained by alkalization are dissolved in alcohol, chloroform, benzene, or toluene, the result in all cases is a bright leaf-green solution.

Characteristics as a Fluorochrome

If acridine orange solutions are prepared in distilled water at pH 6 and the concentrations systematically changed between 1:100,000 and 1:100, a highly characteristic change takes place in the fluorescence spectrum so that the subjective impression of the fluorescence colors when examined under the filtered light of a quartz lamp is very different (cf. too Plate I, 1). Table 22 shows the results of such a series of tests.

Table 22

Konzentration	- Fluoreszenz	(1) Bemerkungen
1:100 000	(2) laubgrün 23 la ++	
1: 80 000	(2) laubgrün 23 na ++	(7) entspricht annähernd der Konzentration im lebenden Plasma
1: 10 000	(2) laubgrün 24 na ++	
1: 8 000	(3) gelb pc grünlich ++	
1: 1 000	(4) gelb 3 na ++	
1: 800	(5) kreß 4 na ++	
1: 600	(5) kreß 5 po ++	
1: 300	(5) kreß 6 pc ++	
1: 200	(5) kreß 6 pc ++	(8) schön gelblichrot
1: 100	(6) rot 7 pc ++	(9) entspricht der Konzentration im toten Plasma

1 - remarks; 2 - leaf green; 3 - yellow pc greenish; 4 - yellow; 5 - cress; 6 - red; 7 - corresponds approximately to the concentration in living protoplasm; 8 - by this time yellowish red; 9 - corresponds to the concentration in dead protoplasm

Acridine orange solutions fluoresce leaf green in the strongly diluted region between 1:100,000 and 1:10,000. Between 1:10,000 and 1:5000 this leaf-green fluorescence gradually becomes greenish yellow. Between 1:5000 and 1:1000 the green fluorescence color is lost and 1:1000 solutions in distilled water fluoresce yellow. Between 1:1000 and 1:500 the color is a reddish orange-yellow and, finally, a 1:100 solution fluoresces deep copper red.

The pattern is that with increasing concentration of the solution, the center of gravity of the fluorescence spectrum shifts more and more toward the longwave region (cf. also Plate I, 1, which shows the fluorescence spectra of two striking concentration gradations).

This change in fluorescence colors in relation to dye concentration is known as the "concentration effect" (Strugger, 1940). The reason for it in physical-chemical respects has not yet been adequately investigated. There may be a connection with the reversible polymerization of pseudoisocyanines observed by Scheibe (1938/1939). The concentration effect occurs, as relevant experiments have shown, only when the acridine orange solutions are dissociated. Should a solution exceed pH 9, the effect no longer occurs since the fluorescence color of the acridine orange base molecule is independent of the concentration.

The concentration effect is a completely reversible phenomenon. If red fluorescing, strongly concentrated acridine orange solutions are gradually diluted, they show under the quartz lamp all the color shades from copper red to green.

Influence of the pH Value on Fluorescence

Since the dissociation of basic dyes disappears slowly but completely with gradual alkalization, it frequently happens in the case of basic fluorochromes that a change in fluorescence properties also follows a change in the pH value. If these changes are very strong and striking, a fluorescence indicator is at hand. For acridine orange pertinent data are to be found in Jensen (1938), who recommends acridine orange for titration. He finds the transition region between pH 8.4 and 10.4 and reports that below pH 8.4 acridine orange fluoresces pale green, but above it it fluoresces intense yellow-green. Thus, acridine orange is a one-color fluorescence indicator. Personal studies on the pH dependence of the fluorescence colors of acridine orange solutions (1:10,000) confirmed Jensen's finding. While 1:10,000 solutions at pH 2 to 8 fluoresce moderately leaf green, the color becomes much more intense after pH 8. As cataphoretic measurements in a direct-current field showed, the dissociation of acridine orange gradually decreases between pH 8 and 9. The intensely fluorescing alkaline solutions contain, therefore, the electroneutral color base molecules, whereas the pale green fluorescing, weakly alkaline and acid acridine orange solutions are ultimately dissociated maximally with increasing acidification.

Fig. 12 shows the acridine orange dissociation curve found colorimetrically by Kolbel (1947). If 1:1000 acridine orange solutions are acidified with strongly dissociated mineral acids, the fluorescence color changes into a reddish green. This treatment, according to Kolbel, brings about another change in the dye (further proton absorption) so that the appearance of this red fluorescence cannot be related to the concentration effect. This is evident from the dissociation curve of acridine orange shown in Fig. 11. According to this curve, the dye achieves maximum dissociation in aqueous solution at pH 6.3 so that from this pH value on toward the acid region the curve is horizontal.

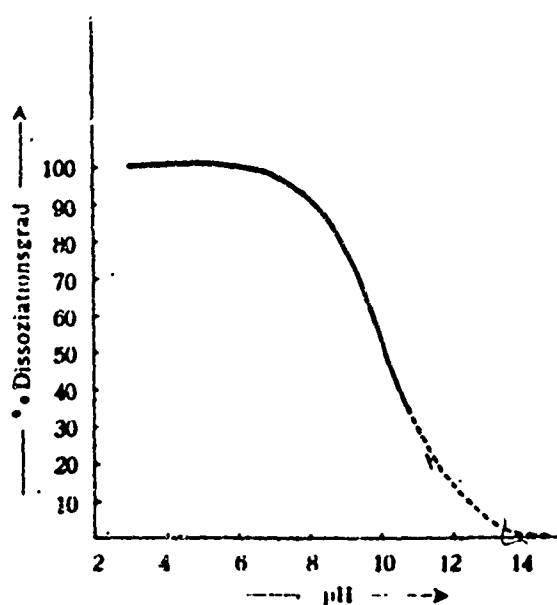


Fig. 11. Dissociation curve of acridine orange in water as determined by colorimetric measurement of the depth of color. The extended part of the curve is measured out, while the broken line is theoretical (Kolbel, 1947).

For biological research, therefore, it must be kept in mind that acridine orange does not significantly change its fluorescence color within the pH range of practical importance, but beyond pH 8 to 9 its fluorescence intensity is considerably strengthened. The color transition to reddish that appears after extreme acidification need not be considered for research involving biological objects.

Table 23

Fluoreszenz		(1) Eigenfarbe
pH	Benzol	Benzol
	(2) Wasser	Wasser
2.23	—	—
	(3) laubgrün 24 na ++	(6) gelb 2 ga
3.81	(4) eisblau 18 te (Mischfluoresz.)	—
	laubgrün 24 na ++	gelb 2 ga
4.83	(5) seegrün 21 la	gelb 1 ga
	laubgrün 24 na ++	gelb 2 ga
5.10	laubgrün 22 la ++	gelb 1 ga
	laubgrün 24 na ++	gelb 2 la
5.91	laubgrün 22 la ++	gelb 1 la
	laubgrün 24 na ++	gelb 2 la
7.04	laubgrün 23 na +++	gelb 1 la
	laubgrün 24 na +	gelb 2 la
7.57	laubgrün 23 na +++	gelb 1 la
	laubgrün 24 na +	gelb 2 ga
7.87	laubgrün 23 na ++++	gelb 1 la
	laubgrün 24 na +	gelb 2 ga
8.06	laubgrün 23 na ++++	gelb 1 la
	laubgrün 23 na (+)	—
10.00	laubgrün 23 na ++++	gelb 1 la
	laubgrün 23 na (+)	—

1 - intrinsic color; 2 - water; 3 - leaf green; 4 - ice blue;
5 - sea green; 6 - yellow

Lipid Solubility

For cathodic dyes the general rule is that the color cations are hydrophilic, while the color base molecules are more or less strongly lipophilic. Therefore, the lipid solubility of a cathodic dye must be characteristically dependent on the hydrogen ion concentration of the dye solution. As long as the dye is strongly dissociated, extraction of the dye with an organic solvent is quite limited or is completely impossible. If the dissociation is repressed, the undissociated color base can easily be extracted in an organic solvent.

For practical investigation of solubility in organic solvents, 1:10,000 acridine orange solutions were prepared with increased pH. These solutions were covered several cm high with toluene or benzene and then vigorously shaken. After the components of the emulsion separated, the distribution of the dye in the aqueous and organic phases was examined in transmitted light and under a quartz lamp. Table 25 summarizes the results.

Although no solubility was observed in the organic phase in the extremely acid region, there was the beginning of a very weak solubility between pH 3 and 4. The solubility of acridine orange in benzene gradually increased as the critical pH 8 to 10 range was approached. From pH 10 on the dye passed quantitatively into the benzene so that the aqueous phase was completely colorless and appeared to be fluorescence-free. These results also confirmed the previous finding that the dissociation of acridine orange is at a minimum between pH 8 and 9 and that the color cations are hydrophilic, whereas the color base molecules are lipophilic.

Fluorochroming of Models with Acridine Orange

Since model tests of simple systems have been found highly advantageous in studying dyeing phenomena in living and dead cells, we have described some model tests with acridine orange which laid the foundation for understanding the fluorochroming of living and dead plant cells. Cellulose fibers and gelatin-toluene were used as models for the cell wall and protoplasm, respectively.

The Cellulose Model

1:10,000 acridine orange solutions were prepared with graduated pH values. Cellulose fibers were dyed for 20 minutes in these solutions and the fibers so treated were washed out in undyed buffer solutions with the same pH. A distinct pattern was then noted in the appearance of certain fluorescence colors. At pH 2.17 the dyed fibers always appeared green even if they were overdyeed the same length of time. Electroadsorption was not so strong, therefore, that a concentration effect and with it a reddish fluorescence color appeared. Beyond pH 3

the fibers began to store the acridine orange vigorously and the fluorescence color became yellow to reddish-yellow accordingly. Between pH 4 and 5 the copper-red color kept gaining the upper hand. Above pH 5 all the fibers were shiny copper red. Past pH 6 the fibers no longer fluoresced red. They appeared green-yellow at pH 6.15 and pure green at pH 10.95. These results cannot be understood until the storage is detected by quantitative measuring tests. Using Duboscqu's colorimetric method, Kolbel (1947) systematically made such measurements in acridine orange solutions of different pH by determining the extinction. The resultant curve is shown in Fig. 12. Acridine orange storage by cellulose is minimal under pH 3.0, but it increases steeply over pH 3.0 until it reaches a maximum at pH 6.7, when it again gradually decreases. Through this curve one can clearly recognize the red fluorescence of the cellulose fibers as the concentration effect.

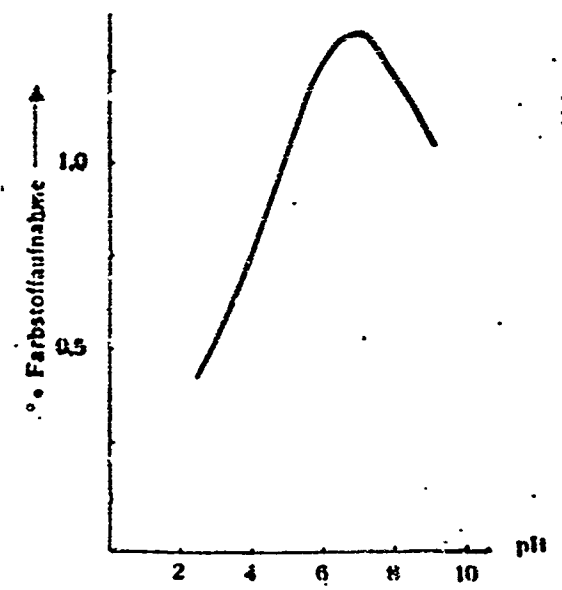


Fig. 12. Colorimetric determination of acridine orange storage by cellulose fibers in relation to pH value. Dye concentration $c = 0.5 \cdot 10^{-3}$ mol/ltr. Ordinate: per cent dye storage relative to the specific weight of the dyed cellulose fibers (Kölbel, 1947).

The storage of acridine orange cations by cellulose undoubtedly involves electroadsorption. The cellulose is negatively charged and with its large inner surface it can bind electrostatically the positively charged color cations. Since cellulose shows a strong decrease in charge in the extremely acid region, it can absorb only a little of the

dye in the extremely acid region. Therefore, such fluoro-chromed fibers can never show a red fluorescence. Cellulose is increasingly charged electronegatively in dye baths over pH 5. It can therefore bind electrostatically more and more color cations with gradually increasing pH. This ultimately results in the concentration-conditioned effect of the red fluorescence color. With increasing alkalization, however, this strong electroadsorption must again become weak at the moment when the electrolytic dissociation of the acridine orange solutions is considerably restricted. This happens when pH 8 is exceeded. If this zone is passed through, only very limited electrostatic adsorption of the acridine orange on the inner surface of the cellulose is possible and the fibers fluoresce just a pale green. The optimum curve shown in Fig. 12 is explained by two limiting factors. In the extremely acid region the slight electrical charge of the cellulose operates as a limiting factor, whereas in the alkaline region the cessation of dissociation of the dye restricts electrostatic adsorption.

The Gelatin-Toluene Model

The composition and preparation of such a model are mentioned on p. 25. Slides with the gelatin-toluene emulsion are placed in dye vats in buffered staining solutions with graduated pH values. They are kept there 50-60 minutes. FM examination of the model starts after the slides are briefly washed in colorless buffer solutions. At pH 2.17 the gelatin fluoresces green, while the toluene drops show no particular fluorescence. At pH 3.25 the gelatin still has a green fluorescence, while the toluene is uncolored. At pH 4.87, on the other hand, the gelatin fluoresces dull copper red, showing therefore a concentration effect, while the toluene drops fluoresce very pale green. At pH 7.9 the gelatin is again pale reddish green, while the toluene drops show an increase in green fluorescence intensity. At pH 10.95 the gelatin is barely stained, at most imbibes pale green, while the toluene drops fluoresce very intense yellow-green.

These observations can be explained by Kolbel's quantitative measurements (1947) of acridine orange adsorption on gelatin in relation to pH value (Fig. 8). Below its isoelectric zone (at pH 4) gelatin protein binds acridine orange weakly or not at all, whereas it is strongly electronegatively charged above its isoelectric zone so that maximum electroadsorption of the acridine orange cations takes place. Toward pH 9.0 dissociation of the dye decreases and there is a corresponding decrease in dye binding on the gelatin. This is paralleled by the appearance of a secondary red fluorescence within the gelatin only in the pH range where electroadsorption is also at a maximum. The behavior of the toluene results freely from the pH dependence of the electrolytic dissociation of acridine orange. With increasing alkalization more and more lipophilic color base molecules can fluoro-chrome the toluene drops a green color. Since the color base molecules do not produce any concentration effect, the fluorescence

colors of the toluene drops should not change significantly with increasing alkalization.

The model experiments show us that cell membranes are fluorochromed a red fluorescence color in the pH interval between 3 and 8 and that this red fluorescence is dependent on electroadsorption of the color cations on cellulose. Gelatin protein displays exactly the same behavior above its IEP as cellulose, while the organic phase of toluene, which was used as an analog for lipid phases, only absorbed an appreciable amount of the dye upon extreme alkalization. It is to be expected, therefore, that protoplasmatic proteins can store acridine orange as cation electrostatically and that this storage has to take place between pH 5 and 8.

Fundamental Experiment on the Upper Epidermis of Allium cepa
Scale for Vital Fluorochroming of Cells with Acridine Orange

The upper epidermis of the Allium cepa scale is a particularly useful object for vital staining experiments and it plays, therefore, a significant role in plant physiology literature. This tissue can be easily isolated by Strugger's (1935) method of preparation. The result is a monolayer membrane of living and mostly undamaged cells which can float on a dye solution, thereby permitting the cuticle-free underside of the cells to absorb the dye. This experimental material is also highly suitable for optical analyses. Moreover, much is known about the protoplasmatic properties of these cells. Strugger (1940) was the first to describe and analyze in detail acridine orange vital staining of the protoplasts in the upper epidermis of Allium cepa scales. The following series of the fundamental experiment will serve as the starting point of our observations.

(1) Series 1. Living epidermis membranes are prepared in a 1:1000 acridine orange solution with tap water at pH 7.3 and allowed to float in small dishes. From time to time after brief washing in tap water they are examined under a blue-light FM.

(2) Series 2. Living epidermis membranes are placed in boiling water for a few minutes to kill them and then dyed in a 1:1000 acridine orange solution with tap water at pH 7.3. Before FM examination, which is carried out from time to time with a blue-light FM, they are washed in clean tap water.

The results of these experiments are shown in Table 24.

After 15 minutes' more dyeing the cell membranes in the living material fluoresce copper red while the cytoplasm and nuclei fluoresce green and yellow-green, respectively. Slight vacuole contraction takes place and the cell sap spaces accumulate the dye so vigorously that the vacuoles fluoresce copper red. Quite a different picture emerges when the dead

cell material of experimental series 2 is kept 15 minutes longer in the dye. The membranes, cytoplasm, and nuclei fluoresce a bright copper red. The cell sap space generally does not fluoresce because it disappears in dead tissue. If the material in series 1 is kept longer in the dye, no significant changes appear after 15 minutes. The cytoplasm and nuclei of the living cells continue to fluoresce green and yellowish green, respectively. A slight increase in intensity does appear after a longer period of dyeing, but a red fluorochroming of the living plasma is impossible despite the excess dye. The duration of dyeing in these experiments was extended to 24 hours. The protoplasts still fluoresced green.

Table 24

(1) Lebende Zellen				
(2) Färbzeit	Membran	(3) Zytoplasma	(4) Kern	Vakuole
15 Minuten	(5) ++ kupferrot	(6) + grün	(7) +++ gelbgrün	(5) + kupferrot
60 Minuten	+++ kupferrot	++ grün	+++ gelbgrün	++ kupferrot
100 Minuten	+++ kupferrot	++ grün	+++ gelbgrün	+++ kupferrot
6 Stunden	+++ kupferrot	++ grün	+++ gelbgrün	+++ kupferrot

(8) Tote Zellen				
(2) Färbzeit	Membran	(3) Zytoplasma	(4) Kern	Vakuole
15 Minuten	(5) ++ kupferrot	++ kupferrot	+++ kupferrot	-
60 Minuten	+++ kupferrot	+++ kupferrot	+++ kupferrot	-
100 Minuten	+++ kupferrot	+++ kupferrot	+++ kupferrot	-
6 (9) Stunden	+++ kupferrot	+++ kupferrot	+++ kupferrot	-

1 - living cells; 2 - dyeing time; 3 - cytoplasm; 4 - nucleus;
5 - copper red; 6 - green; 7 - yellow-green; 8 - dead cells; 9 - hours

The copper-red fluorescence of the cell sap space increases in intensity somewhat and the copper-red fluorochroming of the cell membranes can become deeper after longer periods of dyeing. The dead cells in series 2 show no change in fluorescence color after longer

periods of dyeing. The membranes, cytoplasm, and nuclei remain shiny copper red. It follows from these fundamental experiments that living protoplasm can store acridine orange very rapidly. However, this storage is a limited one because after a much longer period of dyeing with simultaneous exposure to excess dye solution, living protoplasts can never fluoresce copper red, although dead protoplasm is so strongly loaded with acridine orange within the first quarter hour that it fluoresces shiny copper red. This serves to distinguish dead from living protoplasm in a striking fashion.

The coloring of living cytoplasm is absolutely homogeneous. No peculiar structures appear. More precise optical analysis shows that the nuclear reticulum and thus the karyotin or chromatin can store acridine orange electively, whereas the nuclear sap or karyolymph cannot to any significant degree. Moreover, the nuclei store the dye so vigorously that they become quite prominent and after stronger dyeing even appear reddish (Bukatsch, 1941).

Dead cytoplasm appears strongly structured and shiny copper red. The homogeneity is completely lost and rather solid or delicate granulations, flakes, and threads fluoresce shiny copper red. A typical coagulation structure is involved.

The vacuole contraction noted after acridine orange staining is, as experiments have shown (Strugger, 1940), completely reversible. It has only external features in common with the vacuole contraction phenomena occurring on the same object after exposure to lethal influences. The green fluorescence of living protoplasm is particularly beautiful in cells which in a vitally fluorochromed state were plasmolyzed with 0.5 mol of grape sugar or KNO_3 .

In order to become familiar with the patterns of dye storage by living and dead cells, it was necessary to carefully determine the pH dependence of the coloring. Membranes were prepared to study this behavior and while living placed in distilled water for subsequent dyeing. Another batch of membranes from the same bulb scales was soaked in 5% HCl for 5 minutes to kill the cells. The dead membranes were placed in a small dish with distilled water to wash out the hydrochloric acid for subsequent dyeing in buffered dye solutions. These solutions embraced 10 gradations and their pH values were between 1.3 and 9.02. The acridine orange concentration was 1:10,000 and the duration of dyeing 15 minutes in all the experiments. The results are shown in Table 25.

A comparison of the behavior of cell membranes in living and dead material shows considerable similarity in the acid region. Under the discharge point of the cell membranes (about pH 3), the cell membranes cannot fluoresce copper red owing to the absence of cation adsorption. Above the discharge point in the pH 3 to 6.5 range occurs the maximum copper red-fluorescence of the membranes due to electroadsorption.

Above pH 7.0 the membrane coloring in the living material decreases so that the membranes fluoresce only pale green. Membrane coloring is absent in dead material in this pH range, perhaps because of the strong competition of the protoplasts. This behavior is completely understandable when related to the acridine orange dissociation curve and it can be explained in the same way as in the model tests.

Table 25a

(1) Lebend.

pH	Membran	(1a) Cytoplasma	(2) Kern	Vakuole	(3) Bemerkungen
1.33	(4) + grün	—	—	—	(12) teilweise Gewe- be abgestorben
2.60	(5) ++ gelbgrün	—	—	—	—
4.74	(6) +++ kupferrot	—	(4) + grün	—	(13) keine Vakuolen- kontraktion
5.76	(6) +++ kupferrot	(4) + grün	(4) ++ grün	—	—
6.50	(6) +++ kupferrot	(4) + grün	(4) ++ grün	(10) + schwach rotl. grün	(14) Vakuolen- kontraktion ..
6.78	(6) ++ kupferrot	(4) ++ grün	(4) +++ grün	(11) ++ rotlich grün	—
7.17	(7) ++ gelb	(4) ++ grün	(8a) +++ gelblich gelb	(11) ++ kupferrot	(6) —
7.31	(8) ++ grüngelb	(4) +++ grün	(9) +++ gelbgrün	(11) +++ kupferrot	(6) ..
7.50	(4) + grün	(4) +++ grün	(9) +++ gelbgrün	(11) +++ kupferrot	(6) ..
7.72	(4) + grün	(4) +++ grün	(9) +++ gelbgrün	(11) +++ kupferrot	(6) ..

1 - Living; 1a - cytoplasm; 2 - nucleus; 3 - remarks; 4 - green; 5 - yellow-green; 6 - copper red; 7 - yellow; 8 - green-yellow; 8a - greenish yellow; 9 - yellow-green; 10 - pale reddish green; 11 - reddish green; 12 - tissues partly died; 13 - no vacuole contraction; 14 - vacuole contraction

Especially noteworthy is the distinct differences in behavior between living and dead cytoplasm toward acridine orange. While dead cytoplasm behaves like the protein model and therefore in the extremely acid region below the isoelectric zone the plasmatic protein cannot absorb acridine too strongly between pH 2 and 3 and thus fluoresces green, once past the isoelectric zone it can fluoresce intense copper red. This copper red fluorescence gradually diminishes with increasing alkalization and related retrogression of dissociation of the dye. The proteins of the dead cytoplasm are therefore strongly electronegatively charged above the isoelectric zone, resulting in vigorous electroadsorptive storage of the dye cations. This is the reason for the copper-red fluorescence.

Table 25b

Tot(19)

alt	Membran (1)	Zytoplasma (2)	Kern (3)	Bemerkungen
1.11	(1) + grün	(1) + grün	(15) ++ grünlich rotlich	-
2.11	(5) ++ gelblich	(9) +++ gelbgrün	(6) +++ kupferrot	(16) Zytoplasma stich ins Rötliche häufig
3.11	(6) +++ kupferrot	(6) +++ kupferrot	++++ kupferrot(6)	-
4.11	+++ (6) kupferrot	+++ (6) kupferrot	++++ kupferrot(6)	-
5.11	(6) ++ kupferrot	(6) +++ kupferrot	++++ kupferrot(6)	-
6.11	-	(6) +++ kupferrot	(9) +++ gelbgrün	(17) Kern zur Rotfärbung befähigt, aber beginnt erst an der Peripherie stark zu quellen.
7.11	-	(6) +++ kupferrot	(9) +++ gelbgrün	"
7.11	-	(6) +++ kupferrot	(9) +++ gelbgrün	"
7.50	-	(6) +++ kupferrot	(9) +++ gelbgrün	"
8.12	-	(18) ++ kupferrot fahl	(9) +++ gelbgrün	"

1-14 - same as in Table 25a; 15 - greenish reddish; 16 - cytoplasm frequently tinged reddish; 17 - nucleus capable of red coloring but starts on the periphery; greatly swollen; 18 - dull copper red; 19 - Dead

The behavior of living cytoplasm toward acridine orange is fundamentally different. While in the extremely acid region between pH 1.3 and 4.7 no practically useful vital fluorescence is possible, beyond pH 4.7 the cytoplasm has a homogeneous green fluorescence with complete maintenance of life. In the case of a neutral reaction, living cells display an optimally homogeneous green fluorescence of the cytoplasm. Red fluorescence never appears in living cytoplasm no matter how excessive the dyeing. Living cytoplasm behaves much differently from the protein model and dead cytoplasm. Although it may bind the dye cations above its isoelectric zone, adsorption of the acridine orange is so slight that red fluorescence cannot appear.

If we compare the nuclei of living and dead cells with respect to their behavior toward acridine orange, we find patterns similar to those observed with cytoplasm. Here too the dead nucleus takes on a shiny copper-red fluorescence in its karyotin within the pH 2.6 to 6.7 range, whereas the dead nucleus never achieves a copper-red fluorescence, regardless of how strong the over dyeing may be. Under our experimental conditions, over pH 6.7 the nucleus could only fluoresce intense yellow-green. The nuclei in these experiments swelled considerably, perhaps because of the influence of the phosphate, and only after a rather long period of dyeing took up so much dye that they fluoresced copper red. I therefore agree with Höfler (1947) that in abnormally swollen protoplasts copper-red fluorescence of dead protoplasm is slow to appear or does so with difficulty. Investigation of living stained nuclei with an oil immersion shows the following distribution of the dye. Mostly the reticular substance is dyed; the karyotin (chromonema with chromomeres) appears in yellow-green fluorescence clearly differentiated. The nucleoli are also prominent due to specific dye storage. The karyolymph, on the other hand, cannot store acridine orange. Accordingly, the rule is that it is chiefly the DNA-containing components of the nucleus that are able to store acridine orange. In the dead nucleus the same structures, largely coarsened and deformed, fluoresce copper red.

The vacuoles of living and dead cells cannot be compared because in the latter they run out and thus no longer exist. In the living cells acridine orange is stored in ionized form so that the vacuoles fluoresce an intense copper red after strong dyeing. A drop-by-drop or crumb-by-crumb storage could never be ascertained in this object. As Höfler points out, and I myself have confirmed, the behavior of the vacuoles varies from object to object.

Analysis of the Concentration Effect in Living and Dead Cells

It is possible to draw some conclusions on the quantitative side of dye storage from the fact that living cells, no matter how much they may be overstained, produce only green fluorochroming of the protoplasts and that dead cells produce red fluorochroming. It is evident from the in vitro experiments on the fluorescence of acridine orange solutions of different concentrations that red fluorescence of ionized acridine solutions does not appear until the acridine orange is in a 1:100 concentration. Dead protoplasm must therefore be capable of storing the fluorochrome in an approximate order of magnitude of 1:100 because otherwise it would be impossible to explain the shiny copper-red fluorescence of dead protoplasts. Even in a 1:1000 concentration acridine orange solutions fluoresce yellowish. Since the karyotin of living stained nuclei fluoresces yellowish green, the intraplasmatic concentration in vitally stained nuclei should be between 1:10,000 and 1:1000. The dark green fluorescence of the vitally fluorochromed cytoplasm clearly shows that the intraplasmatic concentration of the fluorochrome in cytoplasm is 1:10,000 maximally and 1:100,000 minimally. These considerations make it plain that the red fluorescence of cell membranes, dead protoplasm, and vacuoles depends on a concentration effect.

The following experiments are briefly described to close the chain of proof. If it is a matter of concentration effect in the red fluorochroming of the above components of the cell, the effect must be eliminated by suitably graduated brief periods of dyeing. Technically, this can be done in such a way that living and dead cells remain in a dye bath at optimum pH (about pH 7) for varying periods of time and then examined under the microscope. Corresponding short dyeing periods must in comparison with long dyeing periods then produce a green fluorochroming of the membranes, dead protoplasts, and vacuoles. These investigations are also of methodological interest because a precise knowledge of the absolutely reliable dyeing time is necessary for technical determination of the difference between living and dead protoplasts.

Table 26 summarizes the data on the behavior of live and dead Allium cepa bulb scale epidermis with various durations of steeping in 1:10,000 acridine orange dissolved in tap water. It is evident from Table 26 that with a dye solution of 1:10,000 at least 10-15 minutes of steeping is necessary in order to distinguish clearly between living and dead protoplasts. With 1:5000 and 1:100 concentrations much less time is required.

Table 26a

Lebend (1)

(1a) Lebendzeit	Membran	(2) Zytoplasma	(3) Kern	Vakuole	(4) Bemerkungen
1 Sekunde	(5) grün	-	(5) + grün	(5) + grün	(9) Nukleolen stärker gelbgrün.
2 Sekunden	(5) + grün	-	(5) ++ grün	(6) + gelbgrün	(9) Nukleolen stärker gelbgrün.
4 Sekunden	(5) grün	-	(5) ++ grün	+ bis ++ + gelbgrün	beginnende Vakuolenkontraktion
6 Sekunden	(5) grün	-	(5) ++ grün	(7) + grün (rotlich)	(11) Vakuolenkontraktion etwas stärker
8 Sekunden	(6) ++ gelbgrün	(5) (+) grün	(5) ++ grün	(7) ++ grün (rotlich)	(11) Vakuolenkontraktion etwas stärker
10 Sekunden	(5) ++ + gelbgrün	(5) (+) grün	(6) ++ gelbgrün	(8) ++ kupferrot	(11) Vakuolenkontraktion etwas stärker
1 Minute	(7) gelblich (rotlich)	(5) grün	(5) ++ gelbgrün	(8) ++ kupferrot	(11) Vakuolenkontraktion etwas stärker
2 Minuten	(7) ++ gelblich (rotlich)	(5) + grün	(6) ++ gelbgrün	(8) ++ kupferrot	(12) Vakuolenkontraktion verstärkt
4 Minuten	(7) ++ gelblich (rotlich)	(5) + grün	(6) ++ gelbgrün	(8) ++ kupferrot	(12) Vakuolenkontraktion verstärkt
6 Minuten	(7) ++ + gelblich (rotlich)	(5) ++ + grün	(6) ++ + gelbgrün	(8) ++ + kupferrot	(12) Vakuolenkontraktion verstärkt
10 Minuten	(7) ++ + gelblich (rotlich)	(5) ++ + grün	(6) ++ + gelbgrün	(8) ++ + kupferrot	(12) Vakuolenkontraktion verstärkt
15 Minuten	(7) ++ + gelblich (rotlich)	(5) ++ + grün	(6) ++ + gelbgrün	(8) ++ + kupferrot	(13) Vakuolenkontraktion sehr ausgeprägt

1 - Living; 1a - dyeing time; 2 - cytoplasm; 3 - nucleus; 4 - remarks;
 5 - green; 6 - yellow-green; 7 - yellowish (reddish); 8 - copper red;
 9 - nucleoli more intense yellow-green; 10 - beginning vacuole contraction; 11 - vacuole contraction somewhat stronger; 12 - vacuole concentration intensified; 13 - vacuole concentration very pronounced;
 14 - Dead; 15 - reddish green; 16 - green, border copper red;
 17 - center yellow-green, border copper red; 18 - border of nucleus increasingly copper red; 19 - in interior of the nucleus only yellow-green residue

Table 26b

im (14)

Farbzeit (1)	Membran (2)	Zytoplasma (3)	Kern (4)	Merkmale (5)
1 Sekunde	(5) grün	(5) grün	(5) + + + grün	-
2 Sekunden	(5) grün	(5) grün	(5) + + + grün	-
3 Sekunden	(5) grün	(5) grün	(5) + + + grün	-
4 Sekunden	(5) grün	(6) gelbgrün	(5) grün	-
5 Sekunden	(5) grün	(20) gelbgrün äußlich	(16) grün Rand kupferrot	-
10 Sekunden	(5) grün	(20) gelbgrün äußlich	(16) grün Rand kupferrot	-
1 Minute	(5) grün	(15) gelblich grün	(17) + + + + Zentrum gelbgrün Rand kupferrot	(18) Rand des Kernes zunehmend kupferrot
2 Minuten	(5) grün	(8) kupferrot	(17) + + + + Zentrum gelbgrün Rand kupferrot	(18) Rand des Kernes zunehmend kupferrot
4 Minuten	(5) grün	(8) kupferrot	(17) + + + + Zentrum gelbgrün Rand kupferrot	(18) Rand des Kernes zunehmend kupferrot
6 Minuten	(5) grün	(8) kupferrot	(17) + + + + Zentrum gelbgrün Rand kupferrot	(18) Rand des Kernes zunehmend kupferrot
10 Minuten	(5) grün	(8) kupferrot	(17) + + + + Zentrum gelbgrün Rand kupferrot	(19) Im Innern des Kernes nur noch gelbgrüner Rest
15 Minuten	(5) grün	(8) kupferrot	(8) + + + + kupferrot	-

1-19 - Same as in Table 26a

Purely theoretically these experiments showed that the red coloring of the cytoplasm and nucleus of dead cells as well as the red coloring of the vacuoles of living cells appears only when the dye is sufficiently concentrated. The nuclei in the dead cells were stained red only at the end and penetration of the dye in a higher concentration from the border can be directly observed from the fact that the nuclear borders in the last critical stages of dyeing are already copper red while the interior still has a green fluorescence. There is no doubt then that red fluorescence is caused by increased accumulation of the dye in the protoplasm.

Evaluation of Methods

Fluorochroming of cells and tissues with acridine orange is particularly important in perfecting methods of cell physiology in three respects:

(1) Acridine orange exhibits a remarkable affinity for protoplasmatic proteins and can penetrate into living protoplasm in a very short time. Analysis of this vital fluorochroming has shown that all the essential protein structures of the protoplasts such as the cytoplasm, nuclei, and chromosomes are vitally fluorochromable and they can be examined under the FM with all the desired clarity while remaining completely viable.

(2) Under certain experimental conditions acridine orange fluorochroming makes it possible to distinguish precisely between living and dead protoplasts.

(3) In alcohol-fixed cell material, the IEP of the various cell structures can be determined with acridine orange, thereby providing a convenient new method for determining the IEP of various protoplasmatic structural elements.

Add. 1. Vital fluorochroming of living cells with acridine orange should be carried out with maximally sparing, low concentrations. Also exposure of the object to the staining solution should be as brief as possible. Good results are obtained by following these instructions. We should like to mention here the studies of Strugger and Rosenberger (1944) on vital staining of goat spermatozoa. After careful fluorochroming we were able to keep the spermatozoa as viable in vitro as in the unstained state and artificial insemination showed that such spermatozoa can achieve successful fertilization. Acridine orange in 1:20,000 to 1:100,000 solutions are especially suited for vital fluorochroming experiments. In microorganisms so treated the cytological relations can be followed under the FM during cultivation and growth. Since acridine orange, like all fluorescent dyes, has a photodynamic effect (Tappeiner, 1907; Metzner, 1919, 1924), it is absolutely essential to avoid continuous exposure of the vitally fluorochromed object.

Add. 2. Other methods must be used for precise differentiation of living and dead protoplasm. Concentrated acridine orange solutions must be prepared in order to make available to the dead cell material sufficient amounts of the dye for red fluorochroming of the dead protoplasm. 1:5000 to 1:10,000 concentrations are used for this purpose, depending on the susceptibility of the object. The ratio of the volume of the dye solution to the mass of material to be fluorochromed is also important. One must always be careful to see to it that the fluorochroming solution is greatly in excess of the material to be stained. The pH of the dye solution should lie between 5.5 and 7.8.

Going beyond or failing to reach this optimum region must be avoided at all costs.

Another rather important factor in precise differentiation of living and dead cells is the dyeing time, which varies with the nature of the object used. Different amounts of time are needed for the dye to diffuse into the dead cells and accumulate in sufficiently high concentrations to produce red fluorescence. For example, bulb epidermis in a 1:10,000 solution requires 15-20 minutes of exposure to permit precise differentiation of living and dead cells, whereas in a 1:5000 solution 5-10 minutes are sufficient. Microorganisms with their relatively large upper surface need much less dyeing time. Thus, bacteria in general require 10-30 seconds for thorough dyeing.

Add. 3. Determination of the IEP of amphoteric cell components is possible with one dye alone when acridine orange is used (Strugger, 1947; Nordmeyer, 1947), whereas in other cases such determinations always require a pair of dyes, one basic, the other acidic (Naylor, 1926; Pischinger, 1926; Drawert, 1937). Since amphoteric proteins are positively charged below their IEP, and therefore in the strongly acid region, such protein structures stained in an acid medium should show no electroadsorption of the similarly charged cations. Accordingly, only dye imbibition takes place in these protein structures in an acid dye bath below the IEP and it can be detected by a more or less weak fluorescence. If, however, the IEP is exceeded, the protein gel is electronegatively charged and it can bind the acridine orange cations electrostatically. The consequence of this electroadsorption will be a sharp increase of acridine orange in the protein system so that a copper-red fluorescence will be seen under the FM. Therefore, to determine the isoelectric zones of the individual cell components, the experimental material is fixed by the method of Naylor (1926) and Drawert (1937) with 70% alcohol and then immersed in pH-graduated dye solutions. The isoelectric zone of a given structural element lies in the pH interval in which the fluorochroming changes from green to red. Alcohol fixation is suitable because although it dehydrates the protein colloid, it does not alter the electrical charge.

The Theory of Acridine Orange Fluorochroming of Protoplasm

Living protoplasm can store acridine orange only in a relatively low concentration. It is safe to conclude from the fluorescence behavior of vitally stained protoplasts that the intraplasmatic concentration of this vital dye is of the order of 1:10,000. Dead protoplasm, on the other hand, can bind acridine orange more strongly. Copper-red fluorescence clearly shows that acridine orange is being stored in an intraplasmatic concentration of about 1:100. If living protoplasm is exposed for a rather long period of time to a considerable excess of the dye, as long as it remains viable, storage does not increase and, consequently, there is no change in the green fluorescence

color. The only explanation of this fact is that proteins in living protoplasm can absorb only a little dye. Either the quantity of the free electrical charges which enable the reticular living protein system in the protoplasm to bind dye is very small or the submicroscopic protein micelle is so sterically organized that the acridine orange cations cannot be adsorbed for reasons of space. These ideas fit in well with Frey-Wyssling's thought (1938, on submicroscopic protoplasmatic structures. There is little doubt as to the close connection, established by all investigators of plant and animal cells, between the insignificant capacity of living protoplasm to store dye and the submicroscopic structural arrangement found in such protoplasm. It is only after actual destruction of this submicroscopic structural arrangement that the protoplasmatic proteins are capable of greater binding of dye by adsorption.

The following can be said on the basis of earlier experiments about the mechanism of absorption and storage of acridine orange by living cells. Membrane substances store dye by means of electroadsorption of cations. Proof of this statement comes from the fact that studies on cellulose models (Strugger, 1940; Kölbel, 1947) produced results which can be explained only on this basis. Kölbel also carried out a microcataphoretic investigation of stained and unstained yeast cells (acridine orange). Unstained yeast cells carry a strong electro-negative charge (pH 4-9) in the cell membrane and, consequently, in their outer boundary layer. As a result, unstained yeast cells migrate to the anode. A pronounced pH dependence of the cataphoretic direction of migration was not noted owing to the presence of this electrical membrane charge. If, however, living yeast cells are fluorochromed with acridine orange, the membrane charge is neutralized by electroadsorptive binding of the cations on the membrane. Consequently, above the IEP of the yeast cell protoplasm there is anodic migration of vitally stained cells; below the IEP, cathodic migration. This experiment furnishes precise confirmation of the interpretation of the storage mechanism of acridine orange on cell membranes.

The storage of acridine orange by living protoplasm can, on the basis of the present status of research, also be understood only as cation adsorption on living protein micelle. A comparison of Kölbel's (1947) quantitatively determined acridine orange dissociation curve with the results of vital fluorochroming in various pH ranges clearly shows that a 100% dissociated acridine orange solution can effect a vital plasma fluorochroming. However, this is possible only if, contrary to Höfler (1947), acridine orange cations can be adsorbed by living protoplasm through ionic exchange and electrostatically stored. It is interesting to note that, in contrast with the complete neutralization of the electrical charge of the cellulose membrane by the acridine orange cations, the cytoplasm cannot undergo such neutralization, judging by the cataphoretic investigations of Kölbel (1947). Binding between the negatively charged plasmatic proteins and the positive dye cations thus does not result, as has been shown, in an electrical

neutrality, as is evident from the fact that vitally fluorochromed cells (yeast) up to the LSP_M exhibit an anodic rather than cathodic migration. Kolbel thinks that the reason for this differential behavior of the membrane and the plasma is to be sought in the steric relations. "The dye cation, as compared with the submicroscopic cavities of the plasma, is too large for all the places which carry a free electrical charge to be neutralized."

Acridine orange is one of the few substances that very readily lend themselves to optic identification in living protoplasm. A glance at the existing chemical compounds that behave in similar fashion shows that all the acridine substances like acridine (Strugger, 1941), benzoflavine, acridine red, acridine yellow, rivanol, atebirin, and acriflavine (Hirt, 1939) exhibit the same behavior. They vary, of course, in toxicity and the concentration effect is either weak or quite indistinct. Pyronin₃ (Strugger, 1941) and the fluorescein derivatives should also be mentioned. All these compounds have a similar molecular structure so that it is reasonable to expect that future research will pay closer attention to the relationship between intravital storage capacity and molecular form.

The acridine orange molecule, therefore, is adequate in size and shape to the submicroscopic structure of the protoplasm. The well-ordered protein molecule is completely disrupted as soon as the protoplasm dies. At the same time the proteins coagulate. This change greatly increases the ability to store the dye and such dead protoplasts fluoresce red provided that sufficient dye is available.

Theoretically, it is beyond doubt that acridine orange staining can clearly indicate the specific structural changes in the protein molecules resulting from destruction of the protoplasts. The red fluorochroming of the dead protoplasm with acridine orange is thus experimental proof of the lethal disorganization of the plasmatic protein micellar structures.

The experience gained to date with acridine orange permits an hypothetical explanation of the possible protoplasm states in relation to general biological knowledge. Protoplasm can exist in: (1) a structurally dynamic state or (2) a structurally static state. The structurally dynamic state can be divided into two groups:

(a) State of full vital activity.

Characteristics: The highest arrangement of the submicroscopic protein reticulum prevails. The lipid components, which mark off the submicroscopic reaction spaces in the protoplasm are likewise distributed in optimal fashion. These submicroscopic structures are in a state of constant, well-ordered reconstruction, which is closely associated with

the metabolic processes in the protoplasm. Protein synthesis is continuous and enzyme activity is also well organized.

Phenomena of life: growth, normal, very active metabolism, mobility.

Result of dyeing with acridine orange: The protoplasm fluoresces an even dark green.

(r) State of limited vital activity.

Characteristics: The submicroscopic protein structures like the lipid structures still appear to be well and specifically arranged for viability. However, as a result of age or unfavorable external influences, reversible changes take place in the submicroscopic protein structures. Enzyme activity and metabolism are partially limited.

Phenomena of life: Growth is halted. Metabolism is limited and mobility is more or less inhibited. These protoplasts, however, can again resume full vital activity.

Result of dyeing with acridine orange: The protoplasm fluoresces yellow-green.

The structurally static state can also be divided into two groups:

(a) Reversible.

Characteristics: The highly organized, normally dynamic submicroscopic architecture of the protoplasm is suddenly fixed by dehydration like a snapshot. The characteristic structural reconstruction of active life is halted without significant deformation of the structures.

Phenomena of life: Apparent death or latent life. Metabolism is virtually at a standstill, so too growth and movement. Dry spores, lichen, and moss are in this state. This state can also be induced by sudden freezing without the formation of ice crystals in the protoplasm (Luyet, 1937; Luyet and Gehenio, 1938). After careful supply of water this structurally static state returns to its normal, structurally dynamic state.

Result of dyeing with acridine orange: The protoplasm fluoresces green only after swelling takes place.

(b) Irreversible.

Characteristics: The submicroscopic protein and lipid structures are completely destroyed. The proteins coagulate and coarsening of the protoplasmatic structures can be seen under the microscope. The enzymes are partly inactivated and partly in disorganized activity.

Phenomena of life: No signs of life can be seen. Irreversible death of protoplasm.

Result of dyeing with acridine orange: The protoplasm fluoresces shiny copper red.

There can, of course, be various transitional stages between the limited vital activity and irreversible structurally static state. The death of a protoplast is not a sudden event but a gradual, continuous process. These intermediate stages are irreversible and so they inevitably result in protoplasm death. The collective name of "necrobiotic state" is appropriately applied to this phenomenon.

Characteristics: More or less extensive destruction of the submicroscopic architecture of the protoplasm.

Phenomena of life: No growth, limited metabolism, and absence of mobility distinguish these stages.

Result of dyeing with acridine orange: The protoplasm fluoresces more or less unevenly orange-yellow to reddish orange.

The experience with acridine orange staining to date fits in well with this scheme. Even within a cell local injury to protoplasm after experimental intervention can result in a highly localized change in the dyeability of the protoplasm.

Fluorochroming of Yeast Cells with Acridine Orange

The study of vital fluorochroming of yeast cells has both practical and theoretical significance. From the practical standpoint, a reliable FM differentiation of living, dead, and injured yeast cells presents desirable progress for control processes in the yeast industry. The theoretical interest is derived from intravital analysis of the cytology of yeast cells. Yeast is a particularly favorable experimental object for cell physiology. Unlike the higher plant cells, yeast cells can easily be cultivated after experimental intervention so that viability tests can be undertaken without difficulty after "vital staining".

Strugger (1941) was the first to stain yeast cells with acridine orange. Several years later he concluded an exhaustive study of the subject (1945). Kölbel made a quantitative analysis of acridine orange storage in yeast cells (1947).

Technique of Fluorochroming Yeast with Acridine Orange

Acridine orange fluorochroming of microorganisms has two purposes. It can be used for vital staining of the protoplasm of the microorganisms and for differentiation of living and dead cells. If a microorganism is susceptible to acridine orange, both methods must be developed separately. It can be said for yeast that all my experience to date indicates that there can be no question that yeast cells are susceptible to acridine orange solutions in concentrations of 1:5000 to 1:10,000. Thus, the method of vital staining and the method of differentiation of living and dead protoplasts can be used together in studying yeast cells.

Yeast cells are fluorochromed with acridine orange almost instantaneously. Whether for vital staining or for differentiation of living and dead cells, 30 seconds of exposure are generally all that is needed, i.e., the preparation is ready for examination under the microscope immediately after staining. In a sterile glass test tube, a loopful of the material is dispersed in 1 cc of 1:5000 or 1:10,000 acridine orange solution (prepared with well water or chlorine-free tap water) so as to produce a slight turbidity. One must avoid at all costs having too much yeast for the amount of dye available. This is particularly important when the 1:10,000 concentration is used. A small drop of the dyed suspension in the test tube is then removed with a platinum-wire loop and transferred to a clean slide and covered with a cover glass. In FM studies the excess of dissolved acridine orange does not cause any disturbance worth mentioning because of the thin layer of the preparation and because of the weak fluorescence in the nonadsorbed state.

FM examination of such preparations is best carried out with a blue-light FM. Since the size of the yeast cells is also quite convenient, a simple makeshift apparatus can be used when the requirements are modest. For cytological analysis one must work with a high-performance instrument and an oil immersion with a high aperture.

Fluorochroming of Living and Dead Yeast Cells (*S. cerevisiae*)

Living yeast cells. The protoplast fluoresces deep green. The distribution of the vacuoles and the resultant plasma configuration can be seen with great clarity. The cell membrane, depending on the material used, is more or less clearly fluorochromed. Examined with the immersion objective, it can be recognized as a pale copper-red boundary line. The membrane concentrates the dye cations owing to their

electronegative charge. The vacuole membranes (tonoplasts) stand out as rather intensely green fluorochromed boundary lines. The contents of the vacuoles are generally completely fluorescence-free. (However, I have seen yeast cells that could store acridine orange in the vacuoles. These vacuoles then fluoresced an even copper red or fluorescing copper-red crumbs appeared in them). The cytoplasm fluoresces only an even green. Sometimes a few copper-red shining granules appear in the cytoplasm, but these are probably lifeless inclusion bodies (glycogen, volutin). A 1-2 μ large, round body stands out in one place in the protoplast because of its rather intense yellow-green fluorescence. Careful comparisons with the help of nuclear staining methods showed this body to be identical with the nucleus of the yeast cell. Also published reports (Voigt, 1927; Rochlin, 1933; Badian, 1937) agree in all details with the intravital picture obtained with the FM. Regarding the vital structural peculiarities of the yeast cell nucleus, my own FM observations indicate that besides homogeneously yellow-green nuclei, many cells have nuclei on whose periphery can be seen a sickle-shaped or almost rod-shaped, intensely fluorochromed body. In especially favorable cases I was able to see this body break down into two yellowish-green rod-shaped structures - perhaps the two chromosome equivalents.

The metachromatic bodies earlier described in the literature as volutin are generally fluorochromed copper red with acridine orange. According to the more recent studies of Caspersson and Brandt (1940) and Brandt (1941), we are dealing here with nucleic acid-containing structures that break down and reform in the course of the various developmental stages of the yeast cells.

In judging the vitality of yeast cells by means of acridine orange staining, special attention must be focused on the fluorescence color of the cytoplasm. Copper-red membrane and vacuole coloring should not, on superficial examination, lead to an erroneous conclusion as to the vitality of the cells.

Dead yeast cells. These cells can be distinguished under the FM from living cells by their shiny copper-red cytoplasm fluorescence. Their cytoplasm has a flocculent structure. The distribution of the plasma characteristic of living yeast cells is completely disturbed. In judging dead yeast cells, only the fluorescence of the cytoplasm is decisive. Plate II, 2 shows the gross difference between living and dead yeast cells after acridine orange staining.

Damaged yeast cells. Between the two extremes of living and dead almost all yeast material contains a smaller or larger number of cells whose cytoplasm is fluorochromed more yellowish green to orange-yellow. It is not a matter here perhaps of an incompletely fluorochromed dead cell. Rather the outermost limit of dye saturation must have already been reached in these cases. These intermediary forms are more or less damaged cells whose necrobiotic plasma state can be detected by their rather intense acridine orange storage.

Quantitative Studies Using a Colorimeter

Yeast is especially well suited for quantitative studies on acridine orange storage by means of photometric methods. My co-worker K^ölbel (1947) while maintaining maximally rigorous conditions made countless colorimetric measurements on the quantitative course of acridine orange storage by yeast cells. After K^ölbel confirmed the validity of Beer's law on acridine orange in a concentration range to $1.0 \cdot 10^{-5}$ mol/ltr, he measured acridine orange storage by living and dead yeast cells. Beer's law does not hold above the cited concentration.

0.1 g of pressed yeast material with about 95-99% living cells was finely suspended in 10 cc of water and so mixed with the dye solution that the dye in the above concentration was present in excess. The same procedure was followed for yeast material killed by heat. After 10 minutes of staining, the yeast cells were centrifuged out of the dye solution, the yeast sediment examined under the FM, and the remaining dye liquor measured colorimetrically. Fig. 13 shows the amount of dye taken up by living and dead yeast cells from an acridine orange solution. Examination of the centrifugate shows the plasma of the living cells fluorescing green while the dead cells fluoresce copper red. 1.5% of the dye was stored by the living cells as compared with 5.1% by the dead cells.

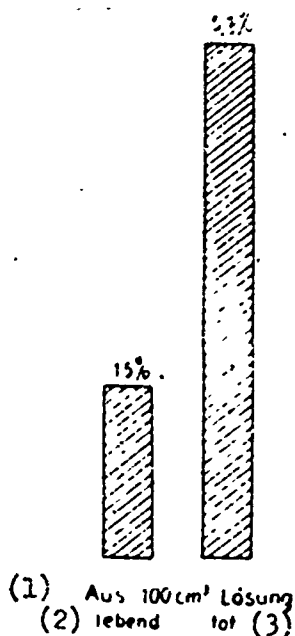


Fig. 13. Acridine orange storage by 0.2 g each of living and dead yeast cells from 100 cm³ of dye solution. Dye concentration $c = 0.5 \cdot 10^{-5}$ mol/ltr. (K^ölbel, 1947)

1-from 100 cm³ of solution; 2 - living; 3 - dead

As a result of Kolbel's studies, the differentiation of living and dead yeast cells is clearly due to the difference in amount of dye stored. After this finding there can be no doubt as to the significance of the concentration effect in the case of multi-color fluorochroming of living and dead yeast cell protoplasts.

Kolbel also studied the relationship between the quantity of dye stored and the time in living and dead yeast suspensions. Fig. 14 presents a curve showing the results. Curve I represents the course of dye storage by a living suspension within an hour, whereas curve II shows the course of dye storage by dead yeast material. On the abscissa are plotted the times of dyeing; on the ordinate, the percentage of dye stored in relation to the total amount of yeast material dyed. This curve is of particular importance for both the interpretation and systematic use of acridine orange dyeing. We can safely conclude from it that living protoplasm can store most of the dye almost immediately and that only a slight increase in storage results from longer exposure. Dead yeast cells can store acridine orange to saturation almost immediately, with only a minimum increase resulting from longer exposure. These findings show that living and dead protoplasts in these microorganisms can be precisely differentiated, provided that an excess of acridine orange is available.

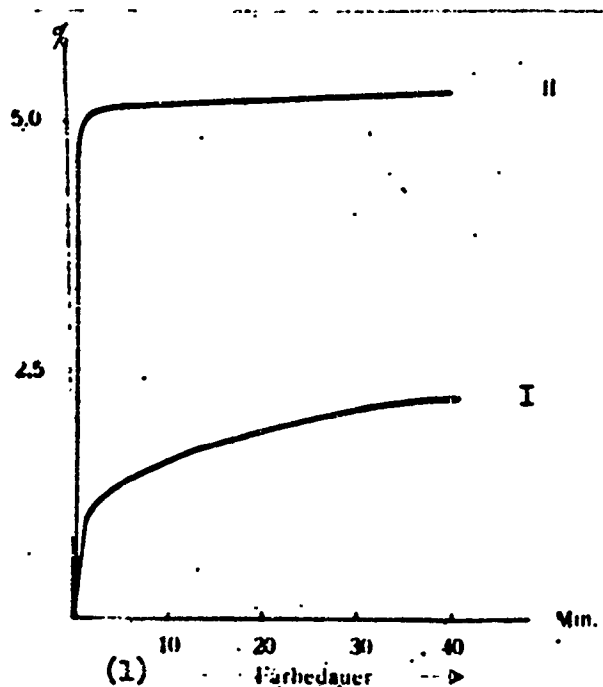


Fig. 14. Quantitative course of acridine orange storage by living (I) and dead (II) yeast cells. Abcissa: duration of dyeing in minutes. Ordinate: percentage of dye absorbed in relation to the weight of the dyed yeast. (Kolbel, 1947)

1 - Duration of dyeing

Use of the colorimetric method made it also possible to determine the amount of acridine orange stored by a single living or dead yeast cell, a matter of considerable importance in FM detection sensitivity of vital dyes in living protoplasm. Kölbel's procedure was as follows. Suspended and dyed yeast cells (0.1 g in 100 cm^3) were counted in a counting chamber 0.05 mm deep under the FM - the same way that blood cells are counted. An Ehrlich counting eyepiece provided the side boundary of the counting field. The length of the side of the field of view was 0.09 mm . By counting 50 space units in each of 4 preparations and then obtaining the mean, the number of yeast cells in a space of 0.000405 mm^3 was found to be 9. In $100 \text{ cm}^3 = 100,000^3$ are $100,000:0.000,405 \cdot 9$ yeast cells = $2,222,222,220$. A single yeast cell then weighs $100:2,222,222,220 = 0.000.000.045 \text{ mg} = 450 \cdot 10^{-10} \text{ mg}$. Since living and dead cells absorb 0.5 and 6.3% of the dye, respectively, then

1 living cell stores $2 \cdot 10^{-10} \text{ mg}$ of dye
1 dead cell stores $28 \cdot 10^{-10} \text{ mg}$ of dye.

Fig. 15 is a graph showing the quantity of acridine orange stored by a living and a dead yeast cell. The results clearly illustrate the high degree of detection sensitivity of a fluorescent dye achieved with the help of a FM. They also reveal the difference between living and dead protoplasm in storage capacity. Living cytoplasm must fluoresce green, dead cytoplasm copper red. From the values so obtained it is possible, by Kölbel's method, to calculate the number of molecules absorbed by a yeast cell and therefore enough to permit FM detection of acridine orange in plasma.

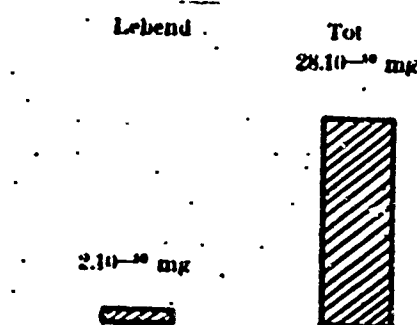


Fig. 15. Graph showing the amount of acridine orange stored by a living and by a dead yeast cell. (Kölbel, 1947)

Since the weight of a molecule is $\frac{\text{molecular weight}}{6.06} \cdot 10^{-23} \text{ g}$, the weight of an acridine orange molecule is $\frac{265}{6.06} \cdot 10^{-23} = 43.7 \cdot 10^{-23} \text{ g}$. Then a vitally fluorochromed cell has $2 \cdot 10^{-10} : 43.7 \cdot 10^{-20} = 4.6 \cdot 10^8$.

mole cells, while a dead cell stained with acridine orange has $28 \cdot 10^{-10} : 43.7 \cdot 10^{-20} = 6.4 \cdot 10^9$ molecules

These figures illustrate in impressive fashion the sensitivity of the FM detection method. It comes between smell perceptibility and the electrosopic detection method for radioactivity. It is evident too that fluorochroming is the only suitable way of solving the vital staining problem.

pH Dependence of Acridine Orange Fluorochroming of Yeast

For practical purposes of differentiating living from dead yeast cells, one must have a precise knowledge of the pH dependence of vital and lethal yeast fluorochroming. I have therefore summarized the 1943 studies on the pH dependence of yeast fluorochroming with acridine orange (Table 27).

Optimum vital staining of yeast protoplasts takes place between pH 4.3 and 10.0. Dead yeast cells do not fluoresce an intense copper red in the strongly acid region to pH 4.3 since the isoelectric zone of the proteins is exceeded. It is not until the weakly acid, neutral, and weakly alkaline region between pH 6.0 and 8.0 that the cytoplasm of dead cells exhibits its characteristic intense copper-red fluorescence.

To distinguish between living and dead yeast cells, it is necessary, therefore, to carry out fluorochroming with an aqueous acridine orange solution at pH 6-8. Acid acridine solutions make a precise distinction between living and dead protoplasts impossible. Hence, for practical purposes it is convenient to work with an aqueous acridine orange solution (distilled or tap water).

Microcultivation Experiments with Vially Fluorochromed Yeast Cells

Three staining types in different proportions, depending on the physiological state of the initial material, can be distinguished in a yeast suspension fluorochromed with acridine orange:

- (1) Yeast cells with protoplasm fluorochromed pure green.
- (2) Yeast cells with protoplasm fluorochromed pure copper red.
- (3) Yeast cells whose protoplasm fluoresces yellowish to orange-red.

Plausible conclusions as to the physiological state of the chief cells in these stages can be made by analogy to the behavior of the higher plant cells and decided finally by microcultivation experiments.

Table 27

Legend (1)

pH	Membran	(2) Cytoplasm	(3) Kern	Vakuole
2.01	(11) grün	—	—	—
3.07	(11) grün	(12) grün	—	—
4.04	(12) grün	(12) grün	(14) grün	—
6.06	(5) rotlich	(14) grün	(6) grünlich	— (+)
7.11	(5) rotlich	(14) grün	(14) grün	— (+)
10.02	(5) rotlich	(14) grün	(14) grün	— (+)

Legend (11)

pH	Membran	(2) Cytoplasm	(3) Kern	Vakuole
2.01	?	(8) gelbgrün	(8) gelbgrün	—
3.07	?	(9) rotorange	(8) gelbgrün	—
4.04	?	(9) rotorange	(8) gelbgrün	—
6.06	(7) kupferrot	(7) kupferrot	(8) gelbgrün	—
7.11	(7) kupferrot	(7) kupferrot	(10) kupferrot-orange	—
10.02	?	(6) grünlich	(5) grünlich	—

1 - Living; 2 - cytoplasm; 3 - nucleus; 4 - green; 5 - reddish;
 6 - green-yellow; 7 - copper red; 8 - yellow-green; 9 - red-orange;
 10 - copper-red-orange; 11 - Dead

A yeast suspension in which the three staining types were richly represented was fluorochromed with 1:5000 acridine orange in tap water present in excess. A microinoculation of a drop of this yeast suspension was made onto a cover slip thinly covered with malt agar, using for this purpose a glass microneedle and micromanipulator, so that 2-10 yeast cells were isolated in the microculture. The preparation was then enclosed in a moist chamber and the color of the cells studied under a blue-light FM and recorded. Care must be taken to keep the irradiation of the vitally fluorochromed cells as brief as possible in order to prevent photodynamic damage. After the color situation is determined, the microcultures can be checked from time to time for growth under an ordinary bright-field microscope with weak illumination. The experiment showed that those yeast cells whose cytoplasm fluoresces an even green after exposure to an excess of acridine orange are fully capable of growth. Their budding rate is no lower than that of unstained control preparations. The cells whose plasma fluoresces copper red can never bud. The cells whose plasma fluoresce yellow to yellow-orange are unable to develop any further.

It is evident from these studies with their numerous supporting experiments that acridine orange fluorochroming ensures clear microscopic recognition of fully viable cells. Also the total dead, plasmatically copper-red fluorochromed cells can be determined with great exactness. The plasmatically yellowish fluorochromed cells are already injured and they must be regarded as necrotically altered.

Investigation of the Effect of Acridine Orange on Yeast Cells

The microcultivation experiments have already shown that acridine orange is not very toxic to yeast cells since they are capable of further normal growth. Evidence of this is provided by gross inspection. If yeast cell, dyed with acridine orange are removed with a loop from a dye-yeast suspension and used to start new cultures on malt agar, such cultures look like nonfluorochromed cultures. It is also possible to add acridine orange to malt agar in the ratio of 1:10,000 or 1:5000 and start yeast cultures on this medium. Here too the cells continue to grow normally and they show in the control under the FM an intense intravital storage of acridine orange in green fluorescence.

The possible toxicity of acridine orange can be determined in the following way. A small portion of yeast cells is removed from the culture with a loop and carefully suspended (test tube method) in 1-2 cc of 1:5000 acridine orange solution prepared with tap water. After 30 seconds of dyeing a microscopic preparation is obtained in such a way that a small drop of the dye-yeast suspension is removed with a loop, transferred to a clean slide, and covered with the thinnest possible cover glass. Examination under the blue-light FM takes place

by means of oil immersion. The living and dead cells are counted in many fields of view by careful analysis of cytoplasm fluorescence. An Ehrlich counting eyepiece with a variable, square field of view is used to facilitate the counting. To avoid accidental error, the largest possible number of randomly chosen fields of view are counted for their content of plasmatically green and plasmatically copper-red cells until a total of 200 cells is counted. According to my experience, this is enough to permit fairly accurate determination of the percentage of living and dead cells. Only those cells are counted as living whose cytoplasm fluoresces an even green. Cells whose cytoplasm fluoresces yellowish or reddish-yellowish, since they cannot grow, are counted with the copper-red fluorescing cells. Therefore, the necrotic stages are also counted as "dead" cells. Several control cultures are started after the vital staining simultaneously with the first counting. The first counting is followed by another 15 minutes later. This is repeated every 15 minutes until a total of one hour of dyeing time is achieved. Then comes the last counting. Several more cultures are started after the last counting. The result of this count is presented in a coordinate system (cf. Fig. 16). Care must be taken to prevent exposure of the dyed yeast suspension to strong light in order to avoid photodynamic damage that could cloud the results. If yeast cells are damaged by treatment with 1:5000 acridine orange solutions, the fact must be reflected in the curve by a decrease in the percentage of living cells in the course of dyeing. This decrease must be accompanied at the same time by a corresponding increase in the dead cells. If, however, the acridine orange solution is entirely nontoxic to the yeast suspension, the percentage of both living and dead cells must, ignoring the usual erratic values, remain at about the same level. Assuming that besides the acridine orange there are no other possibilities of poisoning by the dye solution (contamination of the water, oligodynamic effects of traces of metals, etc.), it is safe to conclude from this experiment that acridine orange is useful for investigating the state of microorganisms. It is evident from Fig. 16 that acridine orange is not toxic to baker's yeast cells even after an hour's immersion in excessive dye solution.

The acridine method in the proposed form is therefore recognized as practically useful for FM differentiation of living from dead yeast cells.

This experiment enables me to return an unequivocal answer to a work by Bucherer (1943), who maintains on the basis of his own studies that living bacterial as well as yeast cells can fluoresce copper red after rather long immersion in acridine orange or in a considerable excess of dye without losing their vitality. Bucherer thought his observations entitled him to criticize the soundness of my method. The absolute weakness of Bucherer's criticism is clearly shown by the results of my experiment. His criticism with respect to yeast is therefore completely wrong, and there is no point in discussing any further his somewhat confused statements.

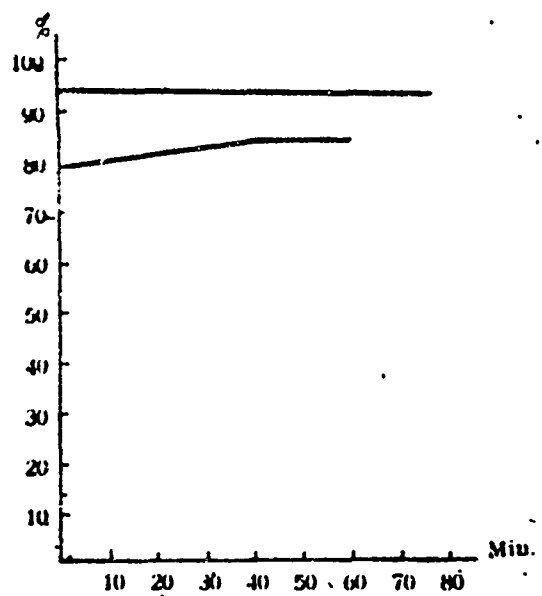


Fig. 16. *Saccharomyces cerevisiae* dyed in 1:5000 acridine orange (with tap water). The two curves illustrate two series of experiments in which the percentage of green fluorochromed and thus living cells was determined by counting at 10 minute intervals. Abscissa: dyeing time in minutes. Ordinate: percentage of yeast cells with green fluorescing protoplasm counted from time to time.

Fluorochroming of Mucor

In my institute Johannes (1947) made a detailed study of the vital fluorochroming of *Phycomyces blakesleeianus* with acridine orange. He found that the living protoplasm of this mold fluoresced green while the dead protoplasm fluoresced red. In the mycelium, depending on the hydrogen ion concentration, of the fluorochroming medium, the membranes were vitally stained in the acid region while the plasma, nuclei, and vacuoles were stained in the weakly acid and neutral region. This was the first time that *Phycomyces* nuclei were stained vitally, thereby rendering this cytologically very difficult object accessible to intravital cytological analysis. *Phycomyces* nuclei exhibit a protistan structure and they can be clearly seen intra vitam in the FM while the mycelium continues to grow. Fig. 17 shows a young germinal mycelium drawn after vital fluorochroming with acridine orange.

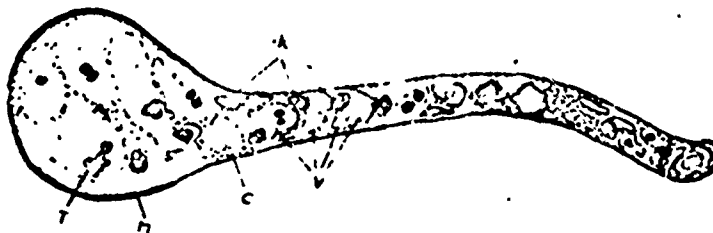


Fig. 17. Young germinal *Phycomyces blakesleeanus* mycelium vitally fluorochromed with 1:50,000 acridine orange (pH 8.4). M = spore membrane copper red, C = cytoplasm diffuse green, K = nuclei green with yellow karyosome, V = vacuoles unstained, T = scattered drop-like storage of acridine orange, copper red in the vacuoles. (Johannes, 1947)

The main rule in vital fluorochroming of such a mycelium is to apply the dye as sparingly as possible to the object. According to Johannes, light intensities of about 200 lux do not cause any significant injury. If the radiation injury is too severe, the death of the protoplasm can be directly seen under the FM in the presence of acridine orange. The plasma is first stained yellow, then orange-red, and finally copper red. Cultivation experiments with these dye stages have shown that the protoplasm is already irreversibly injured when it fluoresces yellow.

Johannes' results with *Phycomyces* spores are of particular interest for practical microbiology. Unswollen spores cannot be stained with acridine orange when alive. On the contrary, only dead protoplasm in unswollen spore material fluoresces copper red, whereas living protoplasm exhibits very weak fluorescence or none at all. The spore membranes have a fluorescence-quenching pigment which must first be dissolved out of the membranes. If the spores are suspended beforehand in a 3% malt solution and stained with acridine orange on a slide from time to time after removal from the suspension, secondary fluorescence increases as the spores continue to swell. These relations are shown in Fig. 18. A red membrane fluorochroming appears 2½ hours after the fluorescence-quenching substance has left the membranes. After 3 hours the plasma begins to fluoresce a diffuse green. After 4 hours the nuclei begin to fluoresce green. After 5 hours the vacuoles enlarge and multiply. After 6 hours the nuclei divide and between the 6th and 7th hours germination begins.

Thus, if acridine orange fluorochroming is used to distinguish between living and dead *Phycomyces* spores, the decision is quickly reached because dead spores' plasma immediately fluoresces copper red whereas live spores' plasma at first does not fluoresce at all. A com-

bined examination of the spores in the bright-field and FM makes such a decision possible. If, however, living spores are to be vitally stained, the procedure must be carried out after a swelling period of 4-6 hours. A 3% malt solution is the best for this purpose.

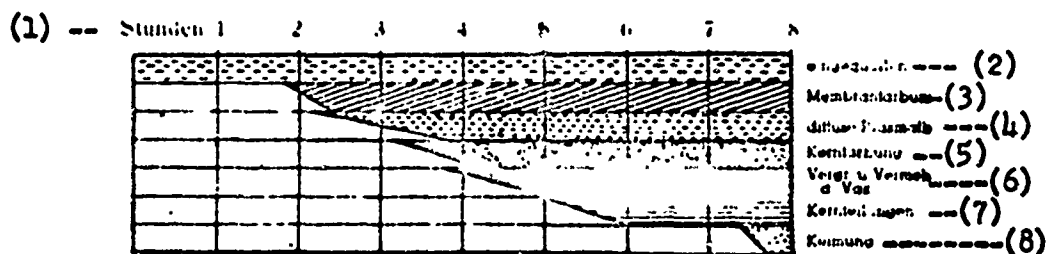


Fig. 18. Spore suspensions of Phycomyces blakesleeianus soaked in a 3% malt solution. Spore samples were removed from time to time and examined on a slide for vital fluorochromability with acridine orange (1:10,000, pH 7.0). The results are recorded in relation to the duration of presoaking. (Johannes, 1947).

1 - hours; 2 - soaked; 3 - membrane staining; 4 - diffuse plasma staining; 5 - nuclear staining; 6 - enlargement and multiplication of vacuoles; 7 - nuclear division; 8 - germination

Johannes thoroughly tested the growth capacity of vitally and lethally fluorochromed spores in microexperiments and demonstrated in all cases the reliability of the acridine orange method of determining the viability of Phycomyces spores.

The difference in staining behavior of the two isogamous genera in copulation that Johannes determined with neutral red (1939) could be repeated with the acridine orange method and even more impressively confirmed because here the red-brown membrane fluorescence contrasts vividly with the green plasma fluorescence.

Acridine Orange Fluorochroming of Bacterial Suspensions

Acridine orange fluorochroming of living and dead protoplasm is highly important in bacteriology. This fluorochrome offers the possibility of developing a vital staining technique for bacteria resting on the fluorochroming of living bacterial protoplasm. Moreover, FM separation of living and dead protoplasm is a systematic way of differentiating living from dead bacterial cells with relative ease. Until now the vitality of bacterial cells could not be determined microscopically. The method of direct evaluation of the protoplast

and its structure under the bright-field microscope, easily applied to larger plant cells by any skilled worker, is useless here because of the smallness of the object. The motility of flagellate bacterial forms sometimes enables the investigator to draw positive conclusions as to the vitality of such bacterial cells, but for obvious reasons it is not suitable for statements on plasma death and, moreover, it is limited only to flagellate forms. While in higher plant cells plasmolysis, with some exceptions, is a valuable method of determining the vitality of a cell, it cannot be used for bacterial cells owing to the smallness of the object and the resultant difficulties of observation. The history of microbiology is not lacking in attempts to correct this inconvenience. Rozicka (1904) worked out a procedure for distinguishing dead from living protoplasm by means of a mixture of methylene blue and neutral red, but it failed to yield reliable results in higher plant cells. Likewise not too reliable is Haurowitz' (1922) methyl green method for distinguishing between living and dead cells (leukocytes). All the attempts to use diachromes to study differences in the behavior of living and dead bacterial cells as a means of distinguishing between living and dead nuclei failed because the bacterial cells are too small to permit reliable detection under the bright-field microscope of the variable dye storage by living and dead bacterial cells. Schumacher (1923/25) proposed the neosalvarsan-silver-malachite-green method. Yeast suspensions are treated for 1 hour with 1% neosalvarsan, washed out 16 to 18 times with the centrifuge, and then mixed with silver nitrate or osmium tetroxide solution. The originally dead yeast cells are stained brown, while the originally living cells do not reduce the osmic acid or the silver nitrate because they do not take up the salvarsan and therefore remain colorless. Contrast staining of such smears with malachite green permits a subsequent green staining of the formerly living yeast cells. Since neosalvarsan is undoubtedly highly toxic and the procedure is quite laborious, it would seem that the method of differentiation proposed by Schumacher is clearly unsuitable.

This is also the reason why the decision as to whether a nucleus is living or dead requires cultivation or animal experiments. Previous bacteriological laboratory technique can by means of these experiments only decide between capacity for growth or virulence, on the one hand, and incapacity for growth and nonvirulence, on the other. It gradually became axiomatic among bacteriologists that inability to grow in a culture or animal experiment is evidence of the vitality of the microorganisms under study while the inability to grow is proof that the bacteria have actually died. Every biologist can agree with the first decision. As to the second decision, however, there are serious reservations. Incapacity for growth may be identical with cell death, but this need not necessarily be so. In themselves bacterial cells can temporarily lose their ability to grow and be regarded as "dead" in both animal and cultivation experiments. Microbiologists have long been familiar with such a change in characteristics with preservation of viability in connection with virulence. Neither type of experiment,

therefore, provides clear-cut scientific proof of the death of bacterial cells. We thus come to the conclusion that until now bacteriologists had no way of distinguishing between living and dead bacterial cells experimentally. The acridine orange method, the rudiments of which were worked out over a period of years on higher plant cells, in principle offers this possibility. It has the advantage of working microscopically so that each individual bacterial cell in a preparation can be evaluated.

From the standpoint of bacterial research technique, therefore, it is undoubtedly rewarding to subject to critical experimental analysis the practicability of acridine orange fluorochroming of bacterial cells. The objectives of such study are three-fold:

- (1) Development of a convenient rapid method of fluorochroming unfixed bacterial suspensions for the purpose of microscopic examination.
- (2) Development of serviceable acridine orange fluorochroming of bacterial suspensions for the maximally precise differentiation between living and dead bacterial cells.
- (3) Vital fluorochroming of bacteria.

Acridine Orange Fluorochroming of Bacterial Suspensions as a Rapid Laboratory Method

In a bacteriological laboratory there is often need of testing pure bacterial cultures in a series for the morphological status of the cells. Also, body fluids like blood, lymph, pus, milk, etc. often have to be tested quickly and in quantity for their content of bacterial forms. A convenient rapid procedure, one requiring a minimum of time for the production of such preparations, has not been available to date. For this purpose smears are usually prepared, dried, fixed, and then treated with one of the usual staining techniques using diachromes. Microscopic examination is possible only after the preparation is completely dry. The procedure usually takes at least 10 minutes. If a procedure could be developed for making usable and clearly differentiable preparations within a minute, it would represent major progress in laboratory practice.

Acridine orange fluorochroming of living cells can be carried out in a very short period of time and produce usable preparations immediately. After 6 years of experience my laboratory has worked out the following procedure for staining bacteria. A drop of acridine orange solution (1:5000-1:10,000), prepared in physiological saline solution, is placed on a slide with a pipette. A moderate amount of the bacteria-containing material is rubbed into the drop for several seconds with a platinum-wire loop. A cover glass is then placed on top of the mixture. Care must be taken that the bacteria-containing

suspension liquid be present in the preparation in the right amount. To do this, it is recommended that a small portion be taken from the stained material with a loop and several preparations be made with a small amount of the liquid. Dyeing commences 30 seconds later. The finished cover-glass preparation can be examined at once. Study under an intense blue-light FM follows. Any laboratory microscope can be fashioned into a usable blue-light FM for bacteriological purposes according to the instructions on p. 8 ff. A rather weak dry objective is used at first in order to distinguish clearly the individual objects in the preparation. The preparation is adjusted with oil immersion to where the bacteria, under low magnification, are presumed to be.

The images obtained by this rapid fluorochroming method are very clear. Since the dye solution is largely used up by the material introduced, the background is generally dark to black, and against it all the dyed elements fluoresce brightly. The bacterial cells have a green, yellowish, or shiny copper-red color. The shape of the bacteria, solid membrane formations and their content can be clearly discerned, something that never happens when the usual smear preparations are viewed in a bright field. Solid membranes or mucous capsules, if present, usually fluoresce copper red. The protoplasmatic structure can be clearly distinguished, so too any inclusions, vacuoles, or spores. Since vital staining of the protoplasmatic body of bacteria is certain, colonies can often be seen in motion.

I should like to emphasize that technically this rapid dyeing procedure does not ensure perfect differentiation of living from dead cells. With careful measurement of the amount of bacteria introduced, it provides at most a rough idea of the vitality of the bacterial suspension.

The useful range of acridine orange rapid fluorochroming will be briefly indicated below.

This dyeing technique has been found especially useful in my laboratory for regular checking of pure bacterial cultures. Changes in the form of the cultivated bacteria as well as any contamination with other germs are very quickly and reliably detected with the help of this technique. It has also been effectively used to investigate bacteria-containing putrescent fluids and mucus.

The method produces quick and unequivocal results in an investigation of the blood of a sick animal for the presence of bacteria. For this purpose a drop of blood is mixed on a slide with the same size drop of 1:10,000 acridine orange solution prepared in physiological saline solution. The untreated specimens prepared from this mixture are examined under a blue-light FM. The red blood cells of mammals are lightly stained with acridine orange so that they do not appear changed under the FM. The white blood cells are stained bright green with acridine orange so that they appear as brightly shining structures

even when viewed with a weak objective. If bacteria or other causative agents of disease are present in blood serum or in the leukocytes, they stand out with their shiny green or copper-red fluorescence. No disturbance of the microscopic bacteriological blood examination by the large mass of red blood cells occurs with this procedure. Disease germs in the blood are less likely to be overlooked by the attentive observer than when the usual smear preparations are used. Thus, I was able to observe clearly druse (lit. gland) streptococci in blood.

The acridine orange rapid fluoro-chroming method is very well suited for detecting trypanosomes in blood (cf. Strugger, 1947). A small drop of 1:20,000 acridine orange solution is prepared with physiological saline solution. A little blood freshly drawn from the experimental animal is mixed with the dye solution and then covered with a cover glass. FM examination reveals a splendid picture. The erythrocytes do not appear, as mentioned above, and therefore cause no disturbance. In strong contrast the leukocytes fluoresce yellow-green. Even with weak magnification the trypanosomes stand out as they float about the preparation vitally stained. The fact that this contrast makes it possible to detect the microorganisms (Trypanosoma brucei) even with weak magnification should be a great help in early diagnosis of diseases caused by them. If such specimens are examined with an immersion lens, the fine structure of the trypanosomes can be clearly seen intra vitam. The cytoplasm fluoresces a pale green. It is quite homogeneous. The nucleus stands out as a bright green stained, somewhat unclearly structured body. The karyosome can still be distinguished. Volutin inclusions fluoresce copper red and they are quite prominent. The flagellum in an optically very favorable place in the specimen fluoresces green, provided, of course, that it moves quite slowly. The rod-shaped blepharoblast fluoresces an intense yellow-green. The continued motility of vitally stained trypanosomes in these specimens is astonishing. The motility of the microorganisms is still readily apparent even after 15 minutes' staining and after intense irradiation in the FM.

The practical usefulness of staining milk centrifugates with the acridine orange method has been demonstrated over the years. The bacteria centrifugated off together with the leukocytes are clearly visible.

My thanks to Professor Schönberg, Director of the Institute of Nutrition, Hannover Veterinary College*, for an original report on his latest results in the field of FM milk research. I quote from this report:

* Cf. F. Schönberg (1946), F. Graf (1938), S. Strugger (1943), G. Gohde (1944), and H. Jung (1947).

"To carry out cell analysis in a milk sediment and to discover the causative agent of mastitis of cows, vital staining with fluorochromes, especially with 1:5000 acridine orange in Strugger's method, has proven to be superior to the previous method involving fixation of sediment smears in heat or with chemical fixatives such as alcohol. Vital staining of a milk sediment with 1:5000 acridine orange is most suited for determining the various kinds of cells and particularly pathological changes in the cells. An examination of such a sediment specimen under the FM reveals not only the individual kinds of cells but also any injury to the nuclei and protoplasm caused by bacterial toxins. Cell analysis by means of the FM provides good indications of possible inflammation of the udder and is of value in prognosticating the course of such mastitis. If it is a question of detecting the causative agents of mastitis, e.g., Galt streptococci, Escherichia coli, enteritis bacteria, or tubercle bacilli, better mixtures of fluorochromes are chosen for vital staining. These include mixtures of acridine orange or acridine yellow and water blue or Congo red to mask undesired fluorescence. The following mixture is recommended: 3 parts acridine yellow (1:5000), 3 parts water blue 6 B extra P (1:5000). The freshly prepared dyes are mixed together and a loopful (1.5 mm) of sediment is placed on a slide and moistened with 6 loopful of the dye mixture and then carefully covered with a cover glass. Staphylococci, streptococci, E. coli, and enteritis bacteria show up clearly. Phagocytosis can be easily observed. It will be noted, however, that FM investigation of milk sediment smears is not as effective for cell analysis as vital staining."

An auramine solution is undoubtedly best for staining tubercle bacilli. If these bacilli are suspected of being present (in a milk sediment: lymphocytes, epitheloid cells, occasional Langhans' giant cells), Hagemann's procedure is used. Heat-fixed milk sediment smears are stained with 1:1000 auramine (Bayer) in distilled water with 0.5% carbolic acid for 15 minutes without heating. They are then rinsed in distilled water and differentiated with alcohol to which 4% table salt and 4% hydrochloric acid have been added. This is followed by rinsing in distilled water and drying.

The acridine orange method is likewise recommended for investigating pus. The purulent discharge of a gonorrhea patient can be quickly and reliably tested for gonococci. All other bacteria like streptococci and staphylococci can be very rapidly observed under the microscope. Phagocytosis can also be traced by means of this method.

Sputum and other mucus have been effectively studied in our institute with this rapid method. In the sputum of tuberculosis patients the tubercle bacilli are stained either green or red and they naturally cannot be distinguished from other similar forms by the acridine orange method.

Acridine Orange Fluorochroming to Differentiate Between Living
and Dead Bacterial Cells

On the assumption that bacterial cells behave like higher plant and yeast cells, the acridine orange method was employed, for the first time in bacteriology, to permit microscopic differentiation between living and dead bacterial cells. A testing of this assumption and a careful, experimentally based analysis of all possible disturbing factors is, of course, absolutely essential before the method can be deemed reliable.

The bacterial cell, in general, consists of living cytoplasm. The question of a distinct membrane in many cases is still moot, but all cytologists are agreed that every bacterial cell has a distinct membrane that is chemically different from the protoplasm. Many bacterial forms have rather dense membranous envelopes, easily seen under the microscope, which are either compact or readily tend toward the formation of a gelatinous substance. The formation of capsules and gelatinous envelopes in zooglea-forming microorganisms belong to this category of bacterial types. In general, however, most bacterial forms have an extremely delicate membrane. The formation of membranes, capsules, and gelatinous envelopes is an important matter in using the acridine orange dyeing technique to distinguish between living and dead bacterial cells because due to the small size of the protoplasmic body the fluorescence color of the protoplasm can be much more easily disturbed or masked than in the case of larger plant cells because of the great storage capacity of the membrane. If the living protoplasm of a small bacterial cell fluoresces green, it is possible to use acridine orange for the following optical images in the case of variable membrane thickness and favorable adsorption relations of the membranes.

(a) Assumption: The living plasmatic body is fluorochromed green. The cell membrane is very weakly developed. Result: Although this weakly developed membrane can store the acridine orange cations so intensely, like the higher plant cells, that the membrane in particular must fluoresce copper red, nevertheless the green fluorescence color of the protoplasm is not disturbed because the disturbing red fluorescing membrane layer is too thin. The green fluorescence of living protoplasm can also be clearly evaluated in such cases by very strong overstaining; so too the red fluorescence of dead protoplasm.

(b) Assumption: The cell membrane is much more developed than hitherto assumed. It is either compact or gelatinous. This rather dense bacterial cell membrane or mucous membrane can store the acridine orange cations so intensely that it fluoresces copper red. Depending on the size of the protoplast, the green fluorescence of the living protoplast will in such cases be disturbed. The superimposed red fluorescence of the cell membrane combines with the green fluorescence of the protoplasm to produce a dirty yellowish-greenish to reddish color.

These complications, however, do not in principle prevent evaluation of the protoplasmatic fluorescence.

(c) Assumption: The cell membrane of certain bacterial types is very dense and compact. The protoplasmatic mass is unusually small and surrounded by the dense membrane. Result: The membranes are stained a shiny copper red, especially in the presence of excess dye, and they completely conceal the undoubtedly existing green fluorescence of the living protoplasts. The combination of red and green fluorescence results in complete obscuring (quenching) of the plasma fluorescence in the interior of the bacteria. Only the intense red fluorescence of the cell membrane on the periphery can be detected.

It is evident from these considerations, which are based on practical experience, that a dense membrane plays a definite role in the acridine orange staining of certain bacterial types. This "membrane effect", as I call it, actually appears often in most gram-positive forms and it must be seriously considered when using the acridine orange dyeing technique to distinguish between living and dead bacterial cells. In discussing the special investigations, these phenomena will be examined in more detail.

Vacuoles, which behave differently from living protoplasm after staining, can be regarded as another possible cause of optical disturbance of acridine orange staining of bacterial cells. Even in yeast cells, which are much larger than bacterial cells, the vacuoles occasionally stain intensely. Then one can observe both a homogeneous green fluorescence of the protoplasm and an intense red fluorescence of the vacuoles. If such yeast cells are examined under the FM with low magnification, they seem in general to fluoresce red, especially when observed superficially. Only careful study with an oil immersion lens clearly shows that it is not a question of dead yeast cells whose cytoplasm has to fluoresce red, but rather of the cytoplasm fluorescing green, with only the vacuoles exhibiting a red fluorescence. Since the overall size of bacteria is much smaller than that of yeast, these phenomena must be highly inconvenient in evaluating cell vitality by means of the acridine orange dyeing technique. On the basis of my many years' experience with numerous bacterial forms I can say, however, that such disturbances are of little consequence in most bacterial forms. This happened only in the case of living spirilla. Here the parietal cytoplasm and the transverse segments of protoplasm lying between the vacuoles were fluorochromed a homogeneous green, while the vacuoles arranged in rows exhibited a bright red fluorescence. A superficial examination of such spirilla might give one the impression that they fluoresce red through and through. Owing to the favorable size of the spirilla, the distribution of the dye can be easily traced.

Bacterial protoplasm, it was found empirically, exhibits exactly the same behavior toward acridine orange that yeast and higher plant cells do, as described above. The green fluorochroming of the cytoplasm

that appears in the presence of excess dye and the red fluorochroming appearing under the same conditions takes place in bacterial protoplasts with the same precision as in all other plant objects. This fact is the main rationale for the use of acridine orange fluorochroming to evaluate the vitality of bacterial cells. In order to work with clear ideas, we propose to speak of the following secondary fluorescence phenomena evoked by the dye: (1) plasma fluorescence, (2) membrane fluorescence, and (3) vacuole or inclusion body fluorescence.

The plasma fluoresce noted in the presence of excess dye is the criterion of cell vitality. The membrane and vacuole effects must be constantly borne in mind when critically analyzing the individual bacterial types. If, however, a bacterial form was extensively tested on the basis of these relations, the acridine orange dyeing technique can be used to evaluate vitality in conjunction with a suitable and likewise critically tested experimental method.

Two things are particularly important in working out a practicable dyeing technique to distinguish between living and dead bacterial cells. First, as already explained for metaphyte and yeast cells, an excess of dye is absolutely necessary. It is evident from the quantitative colorimetric determination carried out by Kölb^{el} (1947) in my institute that dead protoplasts can store 14 times more acridine orange than living protoplasts. The excess dye must therefore also be sufficient in the event that all the microorganisms in the dye bath are dead. Second, both the dyeing medium and the dye itself should show no significant toxic effect at least within the first 5-10 minutes. Otherwise the dyeing method itself can result in falsifying the actual status of the bacterial population. Both problems are so important for the successful use of the technique that they must now be discussed separately and in detail.

Add. 1. The presence of excess dye cannot be ensured by dyeing in a drop on a slide, for the measurement of the bacterial mass with an inoculation loop is too coarse. We therefore recommend the use of a larger amount of dye (1 cc) in a small test tube, preferably one with an inner diameter of 0.9-1 cm and 4-6 cm long. A moderate amount of bacterial material is introduced into the test tube with a loop. The use of a rather dense bacterial mass is to be avoided because there is no guarantee of having excess dye. Besides the highly significant relationship between the quantity of bacteria introduced and the volume of the dyeing liquid, the concentration of the acridine orange solution is another extremely important factor. Experience has shown that if the above instructions are followed, a 1:10,000 concentration is generally sufficient. However, if the bacterial mass is large, a 1:5000 concentration must be chosen.

Add. 2. With reference to toxicity of the dye, two possibilities must be kept in mind. One, acridine orange can by itself injure the cells, especially if used in a relatively high concentration. Two,

injury due to hypotonicity or to contamination of the solvent can also influence the results.

The following is to be noted on the toxicity of acridine orange. Through my studies on metaphyte cells I was able to show that acridine orange is one of the least toxic vital dyes that we biologists have. However, since individual bacterial cells cannot be reliably compared as to their resistance physiology, testing with acridine orange is necessary in principle before reaching a definite conclusion regarding the practicability of this dyeing method. It was experimentally demonstrated that none of the bacterial forms investigated to date suffers any impairment of viability or virulence after vital dyeing with acridine orange. Any injury resulting from prolonged immersion is to be ascribed to the otherwise unfavorable properties of the dyeing medium rather than to the effect of the dye proper.

The following methods were used to determine the toxic effect of acridine orange on a variety of bacterial forms and to assess the status of the vitally fluorochromed bacterial cells: (1) macrocultivation of vitally fluorochromed bacteria on undyed nutrient media; (2) microcultivation of fluorochromed bacteria on undyed nutrient media; (3) cultivation of bacteria on or in dyed nutrient media; (4) quantitative testing of the vitality of bacterial suspensions after prolonged dyeing.

Add. 1. Gram-negative bacteria were dyed for 5-10 minutes with an excess of 1:5000 to 1:10,000 acridine orange dissolved in physiological saline solution. FM examination of material removed from the freshest culture possible revealed that the protoplasm of more than 90% of the cells usually fluoresced green, the rest copper red. This showed that the vast bulk of the bacterial suspension was living. Careful comparative bright-field studies and FM examination of the fields of view revealed the important fact that all the cells in the suspension had stored the dye. In the course of years of observation not one cell failed to store the dye. This presumably important fact was repeatedly confirmed. Dyed bacteria were removed with a loop from the suspension and inoculated onto nutrient agar or into broth. Through this manipulation the fluorochromed bacteria were transferred to fresh dye-free nutrient media where they were able to keep on growing. In comparison with the undyed controls that were inoculated at the same time, hundreds of experiments showed that such vitally fluorochromed bacteria continue to grow quite normally. FM examination of the bacterial cells grown in experimental cultures revealed that in the course of multiplication green plasma fluorescence becomes weaker and weaker and gradually disappears. This is wholly understandable in view of the growth and the lack of a fresh supply of dye.

All the extended experiments on countless bacterial forms showed clearly that up to 10 minutes of fluorochroming with acridine orange does not inhibit growth if fresh bacterial material is used. Animal experiments with pathogenic germs (erysipelas and tuberculosis) yielded

the same result. There was not the slightest difference in the course of the disease between animals infected with vitally fluorochromed bacteria and the controls infected with undyed material from the same culture. Thus, vitally stained bacterial cells completely retain both their capacity for growth and virulence.

Add. 2. The viability of bacterial cells dyed with acridine orange can be demonstrated only by means of a microculture. If green fluorochromed bacterial cells can be checked with a FM for their fluorochroming in a microcultural specimen after inoculation and later their growth is traced under a bright-field microscope, the chain of proof can be regarded as closed by direct microscopic control of the process. Such an experiment is not difficult for bright-field observation, but the previously available methods have to be modified for FM observation. Apart from the physiological suitability of the nutrient medium, FM observation from the optical standpoint requires the thinnest possible cultural substrate, one with no significant natural fluorescence. Since it is desirable to have the maximum fixation of otherwise easily moved bacterial cells or cells with spontaneous movement, the nutrient substrate has to provide for good adherence of the cells to be cultivated. Meat agar cannot be used for optical reasons. It has, in addition, the great disadvantage that dead and consequently copper-red fluorescing bacterial cells gradually lose their color on dye-free meat agar, perhaps because of adsorption displacement. Hence, it is not always possible to distinguish between plasmatically green and plasmatically red fluorochromed bacterial cells on this substrate. Moreover, motile bacterial forms are so motile in the thin layer of liquid on the agar that they float away.

Medically pure gum arabic dissolved in meat broth (1:2) was found to be a useful substrate for such microcultural analyses. A slight addition of 1:10,000 acridine orange does not cause any appreciable optical disturbance and it helps to preserve for some time the copper-red plasma fluorescence of dead cells. A drop of this nutrient substrate is spread thinly on a cover glass and allowed to dry under maximally sterile conditions. The cover glass is now ready for inoculation. The bacterial material is introduced into a test tube with excess 1:5000 to 1:10,000 acridine orange (in physiological saline solution) and then transferred with a small loop to the layer of hardened gum arabic on the cover glass. Care must be taken to remove the excess liquid as quickly as possible with sterile filter paper. The cover glass is placed on a highly polished slide with a little water on it and then made tight with vaseline. These cultures can be conveniently examined in a bright field. By a suitable change of the light filter each place in the specimen can be immediately inspected under the FM. Prolonged FM examination is not recommended owing to the high radiation sensitivity of vitally stained bacterial cells. In order to prevent radiation injury, an appropriate place in the specimen is found with moderate bright-field illumination and oil immersion. This is followed by a switch to blue light for FM examination, which should be as brief as

possible. The aforementioned situation of the bacterial cells is restored (?) to the fluorescence colors of the protoplasts. The microspecimen then remains at room temperature unchanged, microscopically adjusted, without light radiation resulting. The specimen is checked from time to time under the bright-field microscope with moderate illumination and the changes in growth observed as recorded with a drawing or microphotograph.

Experiments on Bacillus bombycis showed the following pattern. Three to four hours after inoculation the green fluorescing bacilli with protoplasm remain unchanged under the microscope. Since unstained control specimens exhibit the same behavior, this cessation of growth represents a general phenomenon not resulting from the shock effect of the acridine orange. After 5-6 hours the homogeneously green fluorescing bacterial cells with protoplasm began to divide quickly, whereas the cells with protoplasm copper-red fluorescing showed no such activity at all.

Similar experiments with other bacterial forms produced the same results. Here, as in the case of yeast, the rule is that only the homogeneously green fluorochromed cells are actually capable of dividing. The necrotic intermediate stages, on the other hand, fail to reveal any capacity for multiplication with this procedure. Plate II, 3, 4 are photographs of an experimental series with Bacillus bombycis (cf. Strugger and Hilbrich, 1942).

Microcultures also plainly showed that bacterial cells whose protoplasm fluoresces a distinct green after dyeing with acridine orange are capable of dividing normally without inhibition and thus remaining completely viable.

Add. 3. It was to be expected from the above results that completely viable bacteria inoculated onto a nutrient medium treated with acridine orange would grow. The procedure is a good test of the relative nontoxicity of acridine orange because the dye can work on inoculated cells or on cells inoculated into acridine orange broth throughout the period of growth. Meat agar or meat extract agar plates were prepared and acridine orange added in concentrations of 1:500, 1:1000, 1:5000, 1:10,000, 1:20,000, and 1:50,000. The plates were inoculated in 3 streaks with Bacillus bombycis, Streptococcus, and Bacterium rhusiopathiae suis from fresh cultures. The results are described in Bacillus bombycis after the cultures were kept for 24 hours in a thermostat at 30°.

1:500 Culture started to grow very poorly (cf. Plate II, 5).

1:1000 Culture streaks began to grow weakly (cf. Plate II, 6).

1:5000 Culture started to grow somewhat more rapidly. Inhibition already somewhat less perceptible.

1:10,000 Culture started to grow very well.

1:20,000 Culture started to grow very well.

FM examination of the material from these cultures clearly showed that all the bacilli were fluorochromed. The inoculated bacteria thus stored the substrate dye and continued to grow. In view of the abnormally long 24 hour period of dye action, which is never the case with vital staining, we see the phenomenon of an only relatively weak inhibition effect produced by the acridine orange. Any inhibition effect that is externally apparent ceases between 1:5000 and 1:10,000.

It still might be said of these experiments with a solid nutrient substrate that dye absorption is insufficient in the course of further growth of the cultures. For this reason other series of experiments were run with liquid cultures and dye added simultaneously. Meat broth was prepared in test tubes under sterile conditions with the addition of 1:500, 1:1000, 1:5000, 1:10,000, 1:20,000, and 1:50,000 acridine orange. Bacillus bombycis was inoculated from a very fresh culture and the cultures were placed in the thermostat and examined 4 days later.

Macroscopic finding:

1:500 Almost clear, very little turbidity.

1:1000 Somewhat turbid.

1:5000 Distinctly turbid.

1:10,000, 1:20,000, and 1:50,000 Very turbid.

FM finding:

1:500 Occasional copper-red bacilli found after laborious search.

1:1000 Occasional bacilli, with red fluorescing and green fluorescing protoplasm in approximately the same number. Multiplication surely took place.

1:5000 Bacilli visible in large numbers, with red fluorescing protoplasm in approximately the same number.

1:10,000 Multiplication extensive. Beautiful chains, perhaps 70% green. Few bacilli with red fluorescing protoplasm.

1:20,000 Multiplication very extensive. Beautiful chains, perhaps 80% green.

1:50,000 Multiplication very extensive, only isolated bacilli copper red.

" Similar experiments were carried out with Bacterium enteritidis Gartner, and the results were the same. Vital staining of the plasmatically green bacilli was easily ascertained from the spontaneous movement of the cells.

These experiments with stained broth are perhaps the best way we have of determining the relative nontoxicity of our vital dye. They clearly showed that acridine orange in a concentration of 1:10,000 even with continuous action in the nutrient medium, is not particularly toxic and that the bacterial cells whose living protoplasm fluoresces green can continue to grow normally.

Add. 4. Of the greatest procedural significance for the practicability of the acridine orange dyeing technique in quantitative analysis of the viability of a bacterial suspension is the above-mentioned toxicity of the dye solution in which the plasmatically copper-red (dead) and plasmatically green (living) cells are counted. This method can produce useful results for science only when no significant change takes place in the number of living and dead bacteria (due to the effect of the dyeing liquid) during the period of time required for the counting. The toxicity of the dyeing liquid can be affected by the following factors: (1) injury caused by hypo- or hypertonicity (2) injury caused by toxic admixtures present in traces in the distilled water or in other chemicals, e.g., oligodynamic action of metal traces; (3) temperature shock effects; (4) eventual toxic effect of acridine orange; (5) action of light.

To determine the best dyeing medium for distinguishing between living and dead bacterial cells, the following was developed as the standard method on the assumption that living cells fluoresce plasmatically green and dead cells plasmatically copper red under all circumstances. With this method the proportion of living to dead bacteria is observed continuously for one hour in order to test the eventual toxicity of the fluorochrome or other impurities in the dye solution. If the ratio changes, it signifies injury to the bacteria. If the ratio remains the same, there is no injury at all.

A small amount of the bacterial material is placed with a loop into 1-2 cc of 1:5000 or 1:10,000 acridine orange solution. The latter is normally prepared in physiological saline solution with twice distilled water. Specimens are made from this bacterial suspension every 10 minutes and their content of cells with and without green fluorescing protoplasm is determined.

Since acridine orange, like all fluorescent substances, can exert photodynamic effects on the cells, every effort must be made to exclude light. Our experience has shown that weak, diffuse daylight has little effect on the viability of a vitally dyed bacterial suspension over a short period of time, but direct sunlight or direct illumination with a strong artificial light should be excluded as much as possible.

To prevent light injury, it is recommended in the case of prolonged dyeing or cultivation experiments that vitally stained bacterial suspensions be kept in darkness as much as possible.

An Ehrlich eyepiece with a square field of view is used for the counting. The diaphragm is adjusted to the density of the suspension. In every field of view all the bacteria with uniformly green fluorescing protoplasm and all the bacteria with yellowish to copper-red fluorescing protoplasm are grouped together. The former are counted as living plus forms, the latter as minus forms, for experience has shown that only the bacteria with green protoplasm are completely viable. The procedure is continued until 200 cells are counted. It is important to count as many fields of view as possible in order to obtain a good statistical average for the specimen. The green and red bacteria are calculated in percentages so as to provide a quantitative insight into the vitality of the suspension under study.

The counting procedure is repeated every 10 minutes, the final count being made after 60 minutes of dyeing. The result is graphed in a coordinate system. The dyeing time is laid off on the abscissa; the percentage of living cells in the suspension, on the ordinate (cf. Fig. 19).

When the counting is over, the bacterial suspension floating in the dye is heated in a water bath to 100° for perhaps 5 minutes. A FM check made after the heating must show a quantitative red coloring of all the microorganisms unless they are particularly heat-resistant. This experiment will show whether, in case all the cells in the suspension die, there is enough acridine orange to ensure complete staining of the now dead bacteria.

Thus, this procedure tests the effect of the dye solution on the vitality of a bacterial suspension after an hour's exposure in a quantitative fashion. The toxicity of the solution can be determined directly from the shape of the curve. If the solution is highly toxic, the curve must fall rather steeply. If the effect is very slight or insignificant, the curve is flat to more or less horizontal. If the curve is completely horizontal, there is no toxic effect at all. The prerequisite of success here is that there must definitely be an excess of dye.

This method can be used to test the susceptibility of a number of important bacterial forms to acridine orange. The test must be made before the dye is applied especially to a new bacterial form. The following rule is most important in determining the usefulness of the acridine orange dyeing technique or that of a new solution combination. Since the staining of a bacterial suspension in the presence of excess dye is virtually completed within the first minute, counting of the cells to judge vitality can be started after one minute. This counting should give us a quantitative picture of the status of the bacteria under study. With reasonable technical skill it should take about 5-6 minutes.

It is essential that neither the dye nor the solvent exert any appreciably toxic effect on the bacterial cells during the first 6-10 minutes. If there were such an effect, the result of the counting would not reflect the true status of the bacterial population being investigated.

Thus, within 10 minutes we can determine from the shape of the curve whether there is such a toxic effect or not. If slight, it should be assumed that only the unavoidable death of already damaged cells occurred owing to the change of medium. If the curve is very steep, extreme care has to be taken in using the acridine orange technique. The reason for the steepness of the curve is to be sought either in the unusual susceptibility of the bacterial material to the dye or in an unsuitable solvent or otherwise unfavorable condition. One must then keep on changing the medium until there are better results or until it is evident that the steepness is preserved in all the media due to the toxicity of the dye itself. The shape of the curve from 20 to 60 minutes is of very little interest as far as the practical use of the acridine orange method is concerned because after a rather long period of dyeing one can hardly count on discovering the original status of the bacterial population. From the theoretical standpoint, however, the further course of the curve is of the greatest interest in investigating the toxicity of various media and the eventual toxicity of acridine orange and it will therefore be discussed again in connection with the research here described.

The choice of solvent for the vital dye was found to be an extremely important matter. As I shall later show in the case of Bacterium coli, it certainly makes a difference which solvent is used to test the vitality of a bacterial suspension. For soil bacteria, for example, physiological saline solution has turned out to be wholly unsuitable since these microorganisms are highly sensitive to sodium ions and to the strong hypertonicity of this solution. Chlorine-free tap water or even better fresh well water is the most natural and least toxic medium for the dyeing. Pathogenic bacteria are best dyed with an acridine orange solution in physiological saline which must be mixed with twice distilled water. Ordinary distilled water proved to be toxic because it is oligodynamic. Twice distilled water is prepared in the laboratory from ordinary distilled water by distillation in Jena glass. As to the saprophytes, it is difficult to pass final judgment. Numerous experiments with various solvents must be performed in order to find the best medium in case of need. A sterile meat broth in which acridine orange is dissolved (1:5000) has been found to be highly suitable for many bacteria, saprophytes, and pathogenic forms. However, the broth has the great optical disadvantage that its inherent fluorescence complicates the FM examination. Moreover, since the broth contains large quantities of proteins, the red fluorescence of dead protoplasts is not very pronounced due to adsorption displacement. For brief vital staining in excess dye for the purpose of counting living cells, I recommend in most cases twice distilled water mixed with physiological saline solution despite the slight toxicity that clearly appears within 30 minutes.

Temperature shock effects can be easily avoided by using solutions of the same temperature.

There is no doubt that acridine orange is toxic when used in a very strong concentration (1:5000 to 1:100). In this concentration the dye is eventually injurious to living cells. I am still uncertain whether this injury is due to its composition or to impurities which are unavoidable in the form of metal traces and other admixtures when organic dyes are manufactured. However, this toxicity has no practical effect in acridine orange dyeing because it can be prevented by choosing more favorable concentration ranges.

Vital Staining of Bacteria with Acridine Orange

The third method of using acridine orange in bacteriology involves the most sparing plasma staining possible. Here an excess of dye is deliberately avoided so that one cannot count on a decisive differentiation between living and dead bacterial cells. The dye is never made available in excess for vital fluorochroming and the concentration is as low as possible in order to avoid any cell injury. The purpose of this approach is to be able to analyze under the FM the structure of the cell in as perfect a condition as possible, to study the motility of the bacterial forms, and to perform cultivation and animal experiments with the help of vitally stained bacterial material.

Strugger and Hilbrich (1942) used acridine orange in concentrations from 1:20,000 to 1:40,000 to prepare Bacillus bombycis, Bacillus subtilis, and Bacterium enteritidis Gartner. We were able to observe the spontaneous movement of the protoplasm of the vitally fluorochromed bacilli. If the dye concentration is systematically increased so as to achieve both a differentiation between living and dead bacilli and a maximally sparing vital staining (acridine orange concentration about 1:20,000), it will invariably be observed that the plasmatically copper-red bacilli exhibit no spontaneous movement while the plasmatically green fluorochromed bacilli exhibit distinct spontaneous movement. Repeated observations on a variety of bacterial forms have clearly shown acridine orange fluorochroming to be a clear-cut indicator of the vitality of bacterial cells.

The following data are taken from my still unpublished reports on Spirillum. Spirilla were cultivated in Hottinger broth and in various concentrations of acridine orange dissolved in tap water, stained, and examined under the FM.

If the 1:40,000 concentration is used, all the spirilla appear quite motile, with the protoplasts fluorescing green. After brief heating they lose their motility and even this low concentration is sufficient to make the protoplasts, after they die, fluoresce copper red. I have kept living spirilla up to 6 hours in 1:40,000 acridine orange solution while the motility plus the green plasmatic fluorescence

remained unchanged. Only after suspension of the spirilla 28 hours longer in the dark does a large percentage of the microorganisms become motionless and the protoplasm fluoresce copper red. In view of this result, dyeing of living spirilla with acridine orange in tap water (1:40,000) can be regarded as successful vital fluorochroming.

A 1:20,000 or 1:10,000 acridine orange solution does not render the spirilla motionless immediately; some 15-30 minutes are required. Of course, the plasmatic injuries in the extremely susceptible spirilla appear much sooner than in the 1:40,000. The spirilla are probably the most susceptible bacterial types to acridine orange dyeing that I have ever encountered. In the 1:40,000 to 1:80,000 range the toxic effect is largely excluded so that future cytological research on living spirilla cells can employ this method. Living and dead spirilla can also be distinguished with the help of 1:20,000 to 1:40,000 solutions within 10 minutes. It will be noted that spirilla overstained with acridine orange can store the dye in their vacuoles in a copper-red fluorescence color.

Unlike Spirillum, Bacterium pyocyaneum is quite insusceptible to acridine orange. If prepared from a young culture in a 1:10,000 solution and suspended in physiological saline solution, quantitative determination of the ratio of plasmatically green to plasmatically red bacilli for an hour will show only minor changes. Thus, even in this high acridine orange concentration, living B. pyocyaneum does not appear to suffer any injury. If bacterial material is taken from this suspension and examined under the FM, the living cells will be found to be very motile. In B. pyocyaneum we have an instance of perfect vital fluorochroming combined with clear differentiation between living and dead cells using the same dye concentration.

The fluorochroming of bacterial populations from putrid waters with a 1:40,000 acridine orange solution in tap water is very impressive. These saprophytic bacteria consist mostly of motile spirilla, motile bacteria, and chains of bacilli. The plasmatically green fluorescing bacteria preserve their spontaneous motility. Here too after careful measurement of both the dye concentration and the amount of bacteria introduced it will be seen that only the bacteria with green fluorescing protoplasm are spontaneously motile.

In all FM investigations of vitally fluorochromed bacteria, one must expect some injury to the protoplasts if subjected to prolonged irradiation under the FM. The early symptoms (slowing of motility) can be detected after 2-3 minutes of exposure. Still longer exposure completely halts motility and the protoplasm begins to turn yellow, reddish orange, and copper red provided that enough dye is present. Such injury to vitally stained bacterial cells is best avoided by regularly moving the specimen about at short intervals of time.

An example of similar vital staining of cells is provided by the studies of Strugger and Rosenberger (1944) on goat spermatozoa. The latter displayed varying degrees of resistance to different acridine orange concentrations. If the vital fluorochroming of this material is to be unobjectionable, the dye is dissolved in a glucose-phosphate diluent by Milovanow's method (1:80,000) and applied to the ejaculum. If the temperature is favorable (+8° and above) and the material is kept in the dark, the vitally stained sperm will remain completely motile for two days. The unstained control sperm behaved the same way. Vital staining with acridine orange also had no adverse effect on the preservation of sperm motility in vitro. Fertilization experiments were performed on 27 she-goats with the stained sperm. Twenty-one animals became gravid and they gave birth to healthy lambs. This research of Strugger and Rosenberger clearly demonstrated the vitality of acridine orange fluorochroming by a very rigorous test.

Applicability of the Acridine Orange Method to Various Bacterial Forms

Several experiments showed that the protoplasts of bacterial cells behave the same way toward acridine orange as the protoplasts of metaphyte and fungus cells. That there is a substantial and serious lack of clarity on this matter is revealed by some recently published reports. For example, Bucherer (1943) maintains that living bacterial protoplasts in the presence of excess acridine orange are fluorochromed red. Although his experimental and optical assumptions are somewhat vague and dubious, this sort of contention in the literature is valuable in renewing the discussion on the similar behavior of bacterial and metaphyte cell protoplasts, for the matter has both practical and theoretical significance. Gärtner (1943) and Stickl and Gärtner (1943) investigated a series of bacterial forms using the acridine orange method. In their work, executed with great accuracy, the distinguished authors concluded that gram-negative forms yield perfect results with the acridine orange method. They were able to analyze satisfactorily the effect of disinfecting agents and sulfonamides on such bacteria with the help of acridine orange fluorochroming as compared with cultivation experiments. In the case of gram-positive bacteria, however, the authors' data indicate that the method is not reliable. After comparing the results with electron-microscopic findings, the authors concluded that the structure of living plasma of gram-negative forms is very compact, whereas that of dead plasma is loose. However, living gram-positive bacteria have the same loose structure as dead gram-negative protoplasts. From this the authors deduced that the protoplasm of gram-positive bacteria is so loose that it fluoresces red after vital fluorochroming with acridine orange. This view has far-reaching consequences from the standpoint of biological theory. If Stickl and Gärtner are right, we would have in the gram-positive bacterial forms the only case in all living nature where fully functional and therefore living protoplasm can store a dye in a 1:100 concentration. Since up

to now only dead, coagulated protoplasm with completely denatured proteins have shown such outstanding electroadsorptive binding of a cathodic dye, investigators of protoplasm, if Stickl and Gärtner are right, would be confronted with a truly unique problem, one with revolutionary implications for fundamental biological research.

In what follows we propose on the basis of a few selected examples to examine the matter and try to elucidate the behavior of various bacterial forms toward acridine orange.

Observations on Gram-Negative Bacteria

Bacterium coli will be discussed in detail as a typical representative of the gram-negative forms. Since other gram-negative forms have been found to behave the same way in essential features, remarks on these forms will be brief.

A small portion of a 12-hour - old slant culture of B. coli is removed with a loop and suspended in a test tube containing 1 cc of 1:5000 acridine orange solution (with physiological saline solution in twice distilled water). The relatively high concentration was chosen to ensure the presence of excess dye. FM observation can start immediately. A loopful of the coli-dye suspension is placed on a slide and covered with an extremely thin cover glass. Examination with efficient lenses shows the following details. The bacilli stand out clearly against the dark background. The bulk of the cells fluoresces green. Only a few are bright copper red. This situation persists even after longer dyeing ($\frac{1}{2}$ hour). It is certain that in the presence of a substantial excess of dye most of the coli bacilli from a young culture fluoresce only green. Control cultures started from the stained bacilli will show an optimum growth in 24 hours as compared with unstained control cultures. The cells fluorescing green must therefore be regarded as viable; the cells fluorescing copper red, as dead. Closer cytological analysis supports this conclusion. It is carried out either on a simple untreated specimen or with the help of a liquid paraffin specimen. This provides considerable optical advantages. A drop of liquid paraffin is placed on a slide and a loopful of the bacterial material rubbed into it. After being covered with a cover glass, places can easily be found in which the fluorochromed bacteria are embedded in the liquid paraffin. In this medium all the details stand out more prominently than in an aqueous medium. The green fluorescing cells exhibit above all a uniform green fluorescence of their protoplasts. No differentiation can be observed in these protoplasts. Since experience has shown that acridine orange produces yellow-green fluorescence in deoxyribonucleic acid-containing nuclear structures, nucleoids, according to Piekarski and Ruska (1939) and Piekarski (1940), should be quite visible in view of the uncommonly favorable optical conditions. However, no trace of such formations is to be found; nor were any vacuoles to be seen in the coli cells. Not always, but quite often, a distinctly fluorochromed

cell membrane can be discerned. With the best objective resolution it can still be observed as a delicate red fluorescing border. This delicate membrane cannot disturb in the slightest the green fluorescence of the living protoplasts.

The copper-red fluorescing coli cells are fluorochromed throughout. Therefore, unlike the living bacteria, the protoplast is copper red. However, the protoplasm is no longer homogeneous; it looks granular and flocculent and is clearly coagulated. Moreover, the dead, copper-red fluorochromed bacteria are somewhat larger than the living green ones because they swell.

It is established, therefore, that B. coli is plasmatically fluorochromed and, as a result, precise FM differentiation between living and dead cells by means of the acridine orange method is theoretically and practically possible.

Living coli bacilli suspended in excess acridine orange are killed by heating to 100° (5 minutes) or by adding formalin. All the cells then fluoresce copper red. Control cultures no longer exhibit growth. Also, the dead plasma of the coli cells can be so loaded with acridine orange that it fluoresces copper red. This is not true of living plasma.

Analysis of the toxicity of various acridine orange solutions is important in ascertaining the reliability of the acridine orange method for B. coli. For this purpose 1:10,000 solutions were prepared in the following solvents for dyeing and intermittent counting over an hour's time: physiological saline solution with twice distilled water, meat broth, physiological saline solution with ordinary distilled water (commercial), and twice distilled water alone. Freshly grown bacterial material was removed with a loop from a 12-hour-old culture on slant agar and immersed in 1 cc of each of the dye solutions. The percentage of living, plasmatically green fluorescing cells was determined every 10 minutes under the FM. Fig. 19 shows the end result of this series of experiments. The first count is made after 2 minutes of immersion. Even this first count shows the regular differences in the values in the various acridine orange solutions. The number of living bacilli was higher in the solution prepared with physiological saline in twice distilled water (97%), somewhat less in that prepared with broth (92%), even less in that prepared with physiological saline in ordinary distilled water (90%), and lowest in that prepared with twice distilled water (82%). Although these differences are initially relatively small, they become more interesting if the further course of the toxicity curve is examined. The differences increase with the duration of immersion so that there can be no doubt as to the initial reality of the effects of the medium.

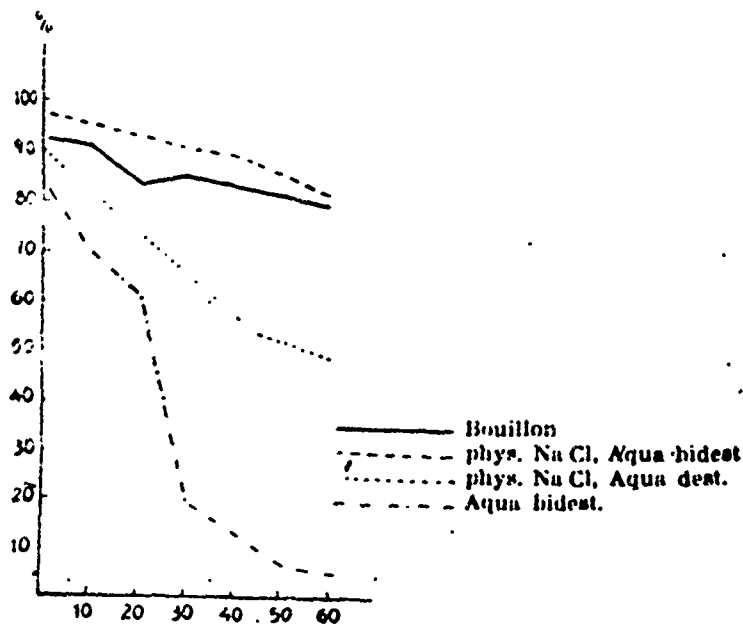


Fig. 19. Bacterium coli dyed one hour in 1:10,000 acridine orange dissolved in a variety of media. The percentage of coli bacteria with green fluorescing protoplasm (living) coli bacteria was statistically determined every 10 minutes. Age of the cultures - 12 hours. Abscissa: duration of dyeing in minutes; ordinate - percentage of bacilli with green fluorescing protoplasm counted each time.

The result is most favorable in acridine orange prepared with physiological saline in twice distilled water. Within an hour the number of living cells only falls from 97 to 82%. In acridine orange prepared with meat broth, the result is equally good. In acridine orange prepared with physiological saline in ordinary distilled water, the percentage falls from 90 to 49.5. The strongest toxicity is exhibited by the solution prepared only with twice distilled water. The drop here within an hour is very big (from 82 to 5%). Control cultures started at the beginning and end of the experiment with twice distilled water showed different appearances after 24 hours. Cultures from the beginning of the experiment did well, producing luxuriant growth, while those inoculated at the end of the experiment had only some scattered colonies.

Physiological saline solution prepared with twice distilled water (second distillation in Jena glass) is thus along with meat broth the most suitable solvent for acridine orange in carrying out

quantitative analyses with Bacterium coli. Ordinary distilled water is oligodynamic in action due to metal traces. Twice distilled water without table salt, on the other hand, injures the cells because of hypertonicity.

This example shows how important it is to find the right solvent for each bacterium before starting systematic use of acridine orange fluorochroming. There can be no "standard formula" owing to the great physiological diversity of bacteria.

The practicability of acridine orange fluorochroming for precise FM differentiation between living and dead coli cells can be considered assured in the light of the above discussion.

What has been reported here on B. coli is equally applicable to other gram-negative forms, judging by my earlier studies on B. paratyphi, B. schottmüller, B. pullorum, B. proteus, B. fluorescens, and B. pyocyaneum.

Cytologically, all these forms display no abnormal behavior as a rule. A 1:10,000 acridine orange solution prepared with physiological saline in twice distilled water permits fairly precise analysis within 20 minutes of immersion. Hence, there is no need to present the resultant curves.

Through the kind cooperation of Prof. Stumpke, director of Linden Hospital in Hannover, I had several opportunities to study with the acridine orange method Micrococcus gonorrhoeae in fresh pus obtained from gonorrhea patients. Dyeing the pus with acridine orange in physiological saline solution (1:10,000) resulted in all cases in clear staining of the extracellular and intracellular gonococci. Since living and dead gonococci can be readily distinguished and since there was no trace of membrane staining, simple acridine orange fluorochroming ensures differentiation between living and dead Micrococcus gonorrhoeae cells. As Dr. Meyer zu Schweicheln in cooperation with Prof. Stumpke's clinic and my institute (1943) demonstrated, gonococci can be perfectly differentiated with acridine orange. A culture of vitally stained gonococci was possible.

Vonkennel and Wiedemann (1944) likewise used the acridine orange method to study living and dead gonococci. It is noteworthy that the authors investigated the toxicity of the dye for gonococci since other acridine compounds, e.g. quaternary ammonium bases (trypaflavine), rivanol) are excellent bactericides. Concentrated acridine orange solutions up to 1:10,000 inhibit gonococcal multiplication. Normal multiplication could not be achieved until a dilution of 1:40,000. Also, gonococci which grew in a substrate to which acridine orange was added in the ratio of 1:40,000 produced green fluorescing, living and further cultivable bacteria after redyeing with acridine orange. For vital staining and for differentiation between living and dead gonococci, however, 15 minutes' dyeing in 1:10,000 acridine orange

proved to be nontoxic so that the gonococci were not influenced physiologically after this time of action. Table 28, which is taken from the above work, clearly shows the usefulness of the acridine orange fluorochroming method in judging gonococci.

Table 28

Material	(1) Acridinorange-Konzentration	(2) Zeit	(3) Fluoreszenzdiagnose		(6) Kulturwachstum
			(4) grün	(5) rot	
Kultur	1:10000	24h	—	++++	—
Kultur	1:40000	24h	++++	—	++++
Kultur	1:10000	15'	++++	—	++++
(7) Kultur 72h auf 5° unterkühlt	1:10000	15'	—	++++	—
(8) Ausstrich durch Hitze fixiert	1:10000	15'	—	++++	—

1 - acridine orange concentration; 2 - time; 3 - fluorescence diagnosis; 4 - green; 5 - red; 6 - culture growth; 7 - 72-hour culture supercooled at 5°; 8 - smear fixed by heat

Vonkennel and Wiedemann (1944) investigated gonococci in pus in the following fashion: "A little pus is obtained from the urethra with a platinum loop and thoroughly mixed on a slide with a drop of 1:10,000 acridine orange solution at pH 7.38 (in Na-K-phosphate buffer). A cover glass is applied and then pressed with filter paper to absorb any excess fluid present on the margin. The specimen is examined immediately."

The constituents of gonorrheal pus then fluoresce as follows. The nuclei of the leukocytes appear green, but they swell considerably after a few minutes. Copper-red nuclei can be seen after somewhat longer dyeing. The granulated plasma fluoresces red. The epithelial cells appear variably red or green. The plasma and nuclei of these cells also exhibit a variable behavior. Gonococci are stained partly green and partly red, depending on the form of the disease. Extracellular gonococci, chiefly those resting on epithelial cells, in general fluoresce exclusively green, whereas intracellular gonococci mostly fluoresce red. Only a few of the latter fluoresce pale green.

Vonkennel and Wiedemann also determined the effect of sulfonamide treatment by means of the acridine orange method. Whereas before treatment of the patient intracellular green and red gonococci and

extracellular only green and therefore living gonococci are present, within a few hours of sulfonamide administration red extracellular gonococci can also be seen. In the course of successful sulfonamide therapy the gonococci suddenly disappear. Sometimes a few extra- and intracellular red gonococci persist. The two authors regard their observation as further evidence of the direct effect of sulfonamides on gonococci. The direct effect of these drugs on living gonococci can also be observed in vitro by means of the acridine orange method.

It appears from these results that the gram-negative Micrococcus gonorrhoeae differs from the gram-positive cocci particularly in the absence of the membrane effect.

Observations on Gram-Positive Bacteria

Staphylococcus aureus (Micrococcus aureus), as a characteristic representative of this group, will be discussed first. Bacterial material from a 12-hour-old culture in full growth is dyed in the usual way in 1:10,000 or 1:5000 acridine orange (with physiological saline solution in twice distilled water). FM examination of the staphylococci under low and medium magnification reveals the unexpected and surprising picture of total red fluorescence of almost all the staphylococci. Parallel cultivation experiments are not in agreement with this and in all cases they show a maximum growth of the inoculated red fluorochromed staphylococci. When these bacteria are examined under the FM and the highest magnification with the best Zeiss apochromatic objective, the reason for the copper-red fluorescence becomes clear. Only a few cocci show solid red coloring in the interior of the cells. In these apparently dead bacteria the entire protoplasm fluoresces red. The bulk of the staphylococci, on the other hand, shows a different distribution of the dye. The solid, frequently capsular membrane of the cocci fluoresces copper red. The interior of the cells appears gray-black in most cases, but in other cases a definitely green fluorochromed protoplast can still be seen through the solidly copper-red membrane. A few cocci whose protoplasm fluoresces a somewhat more intense green are visible among these cells. Such cells are surrounded by a more delicate yet also clearly copper-red stained membrane. Only a scattering of staphylococci have no membrane at all and the plasma fluoresces a pure green. They behave like the gram-negative forms.

The numerous observations on the appearance of a minority of staphylococci with protoplasm fluorescing green can be explained by the fact that quite young cultures, which are vigorously growing, contain many more of these cells than do old cultures. It is reasonable to assume, therefore, that young cocci in a state of rapid division possess only very delicate membranes whose red fluorochroming does not interfere with observation of the plasma fluorescence.

After the cultivation experiments once again showed that almost 96% of the red colored staphylococci had optimum viability, it was fair to conclude that the acridine orange method cannot be used to differentiate between living and dead cells without a thorough scientific reexamination and elucidating of the phenomena.

The following experiment reveals that the bulk of the copper-red fluorescing staphylococci when absolutely dead behave differently with respect to the dye. Heat-killed suspensions were dyed in excess 1:10,000 acridine orange prepared with physiological saline solution in twice distilled water. FM control with the best lenses now shows a uniform picture. All the staphylococci not only have a peripheral membrane coloring but also are solidly copper-red fluorochromed in their interior.

Thus, dead staphylococci are distinguished by the red coloring of the membranes and protoplast, whereas most vitally fluorochromed cocci only have a shiny copper-red membrane fluorescence. The above-mentioned observations on staphylococcal cells whose membranes are delicate or barely developed show, however, that green fluorescence of the living protoplast is theoretically possible. The absence of green fluorescence of the living protoplast or a red fluorescence thereof can therefore not be the cause of the abnormal behavior of most living staphylococci after ordinary acridine orange fluorochroming.

The cause of the abnormal behavior is rather the interference by the red fluorescence of the membranes. This phenomenon is called hereafter the "membrane effect". The volume of staphylococcal protoplasts is relatively very small and the membranes are solidly developed in comparison. The green of the protoplast must be quenched by the red of the membrane complex, for green light and red light produce darkness. It is a matter then of the quenching of existing plasma fluorescence by the strong fluorescence of the membrane. Hence, the interior of such living staphylococcal cells appears more or less dark. The cell membrane, on the other hand, is less solidly developed so the quenching effect gradually diminishes and the protoplast begins to shine through a dull green. With increasing reduction of the membrane the green plasma fluorescence becomes more or more prominent.

This theory requires experimental material and development therefrom of a modified acridine orange fluorochroming technique for staphylococci to permit precise differentiation between dead and living bacteria. For this purpose it is necessary to eliminate electively the membrane fluorescence or to prevent it. From my experience in dyeing the membranes of high plant cells with acridine orange I have adopted the following procedures: (1) extensive suppression of the membrane fluorochroming by alkalization of the dyeing medium; (2) elimination of the asphyxia which greatly promotes membrane staining; (3) elimination of membrane staining by using as indifferent a fluorescence quenching agent as possible.

Add. 1. As is evident from Table 25, the staining of higher plant cell membranes is a purely cation adsorption effect. Above pH 3 the membrane fluoresces copper red. With increasing neutralization or alkalinization of the dye bath, the membrane is optimally stainable copper red up to pH 7 because sufficient dye cations are available in the dye bath. Only when the dissociation of the acridine orange is considerably suppressed by further alkalinization (between pH 7 and 9) does the cell membrane markedly reduce cation adsorption so that it fluoresces yellowish and then greenish.

I endeavored to apply this knowledge to the staphylococci. Staphylococcus aureus was taken from a fresh culture and placed in an acridine orange solution prepared with physiological saline in tap water (pH 7.5-8.5). The result of the dyeing was quite like that described above. The membrane stain became much less intense than that of the control in which the staining solution was prepared with physiological saline in twice distilled water. The green, vitally fluorochromed protoplasm of the staphylococci shone much better through the still red fluorochromed membranes. Thus, this procedure somewhat moderated the disturbing membrane effect.

Unfortunately, the alkalinization procedure is poorly suited to eliminate the membrane effect in practice because only in a relatively narrow critical pH range, with simultaneous elimination of the membrane stain, was it also possible to achieve a reliable red fluorochroming of the dead staphylococcal protoplasts. Since in practice such fine pH intervals are hard to maintain, my efforts to overcome the membrane effect by alkalinization are only of theoretical interest.

Add. 2. I was able to observe the asphyxia effect in higher plant cells vitally stained with acridine orange (1940). Onion skins were immersed for 15 minutes in an acridine orange solution (pH 8.5). They were then carefully dried with filter paper, sealed off from atmospheric oxygen in liquid paraffin under a cover glass, and examined under the FM. Immediately afterward the protoplasts fluoresced a beautiful green, so too the membranes, while the vacuoles in all the cells shone a dull copper red owing to the concentration effect. As the lack of oxygen intensified, the distribution of the dye in the liquid paraffin clearly began to change within 15 minutes. The cell membranes gradually became shiny copper red as the dyeing intensity of the vacuoles decreased. I was also able at the time to reproduce the same effect after transferring the skins predyed in an alkaline medium to acid buffer solutions at pH 5. I formulated the theory of this phenomenon as follows. Asphyxia altered aerobic respiration so that intramolecular respiration of the cells took place simultaneously with acid production in the plasma boundary layers adjoining the membranes. The result of such acidification of the cell membrane is a dissociation of the dye molecule emerging from the vacuole due to the diffusion gradient, and dye cation adsorption on the membrane necessarily follows. Judging from the above experiment, there can be no doubt that a state of asphyxia is favorable to membrane staining in every respect.

It is quite evident from the carbon dioxide production counts of aerobic bacteria that living staphylococcal suspensions exhibit much more intense respiratory activity than do the higher plant cells. The preparation of relatively dense bacterial suspensions in a dyeing medium does not match the natural conditions of life of the staphylococci as found on a surface culture. Consequently, it is fair to assume that staphylococcal cells stained in normal acridine orange solutions soon reach a state of asphyxia, which intensifies the membrane staining.

For these reasons I tried to eliminate the asphyxia by placing staphylococci in a 1:10,000 acridine orange solution prepared with physiological saline in twice distilled water (1-2 cc) while adding at the same time some drops of commercial hydrogen peroxide. A vigorous evolution of oxygen immediately follows introduction of the staphylococcal material into this dye solution. The staphylococci then liberate a good deal of catalase, which decomposes the H_2O_2 . Microscopic examination of this specimen revealed surprisingly that the membrane staining of the living staphylococci was largely eliminated while the green plasma fluorescence was clearly visible. The dead bacteria, on the other hand, fluoresced a solid copper red. Re-examination of an H_2O_2 specimen 10-15 minutes later with cessation of oxygen evolution in the test tube showed that asphyxia had set in and that the membranes were again stained so intensely that the green plasma fluorescence was once more concealed. The H_2O_2 method is only of theoretical interest because the observation conditions in cover glass preparations penetrated by bubbles are quite unfavorable for fluorescence microscopy. The hydrogen peroxide experiment clearly shows however, that the above-described theory of membrane staining rests on correct concepts.

Add. 3. Fluorescence quenching is a pronounced characteristic of many substances. Stained and unstained substances are often capable of completely quenching the fluorescence of certain fluorochromes simply through the presence of their molecules. The following is of importance in doing away with membrane fluorescence by quenching:

(1) The fluorescence-quenching substance must not injure the bacterial cells in any way. Therefore, it has to be physiologically inert.

(2) In the case of living staphylococci, the quenching agent must penetrate only the intermicellar structure of the membrane and quench the red fluorescence there, but it must not penetrate the living protoplasm and quench its green fluorescence.

(3) The quenching agent must penetrate the protoplasts of dead staphylococci only slowly and with difficulty so that their red fluorescence must remain dependably unquenched at least for the first 10 minutes.

Since fluorescence quenching was not hitherto systematically used in cell physiology except in my prontosil studies (Strugger, 1943), the search for a suitable substance that met the above three conditions was quite arduous, and it led to the use of water blue. Water blue is a very coarsely dispersed acid dye with such large particles in aqueous solution that they are unable to penetrate living protoplasts at all and are therefore completely nontoxic and physiologically inert. The particle size is so favorable that the intermicellar spaces of the cell membranes are quickly and surely filled with water blue while the living outer plasma boundary later blocks the dye as an ultrafilter according to Ruhland's permeability theory. The degree of dispersion of water blue is such that it takes 10 minutes for the particles to penetrate the dead protoplasts. Thus, water blue meets the three conditions set forth above.

There are two possible ways of using fluorescence-quenching water blue to eliminate the membrane effect in staphylococci. Water blue can be used simultaneously with acridine orange, or the bacterial material can be first immersed in acridine orange and then examined in a water blue solution of a suitable concentration. The simultaneous action of the two dyes is technically possible, but there are two practical difficulties in the way. One is that the anodic water blue when mixed with the cathodic acridine orange partly separates in flakes so that only filtered solutions can be used and these, moreover, have only limited keeping quality. The second difficulty is that the copper-red plasma fluorescence of dead staphylococci remains unquenched only for the first 10 minutes so that the dyed bacterial suspension cannot be kept any longer. Despite these disadvantages, the use of both dyes simultaneously has proven to be useful in the following modification. Staphylococci are introduced into a test tube containing acridine orange (1:5000) and water blue (1:5000) prepared with physiological saline solution in twice distilled water. The solution must be filtered. If the microorganisms are counted and examined under the FM within 5 minutes from the start of dyeing, young staphylococcal material (15-hour-old culture) clearly shows the expected pattern, one in complete agreement with cultivation experiments. Well over 90% of the staphylococci exhibit pure green plasma fluorescence with total quenching of the membrane coloring. Only a few are characterized by red plasma fluorescence and, consequently, are to be considered dead. After 5-10 minutes' dyeing the dead cells too are largely quenched and they gradually fluoresce yellowish red, yellow, and finally green. The plasma of staphylococci killed by boiling becomes copper red during the first 5 minutes of dyeing, after which it loses its color. Therefore, if the simultaneous acridine orange-water blue method is to be used to judge the vitality of staphylococci, great care must be taken that the dyeing and observation time take no more than 5 minutes.

Preliminary dyeing of staphylococci for 5-10 minutes in 1:5000 to 1:10,000 acridine orange prepared with physiological saline solution in twice distilled water and secondary transfer to 1:5000 water blue

proved to be a much simpler procedure. The advantage of the consecutive method is that vitally stained bacteria can remain unchanged for some time. Small portions of the staphylococci can be periodically transferred to the water blue solution for microscopic examination. Furthermore, a water blue solution provides very favorable optical conditions as a medium for FM examination because the field of view is completely dark. The successive method is applied as follows. Staphylococci are transferred with a loop to a 1:5000 acridine orange solution with physiological saline solution in twice distilled water. After 5 minutes' immersion the centrifuge test tube is placed in the centrifuge and after 5 minutes' spinning (2500-3000 revolutions) the dyed microorganisms are allowed to settle. The dye solution is poured off except for a few drops so that a very dense, vitally stained bacterial suspension is available for further manipulations. The staphylococcal material is now removed with a loop from the sediment, mixed on a slide with a small drop of 1:5000 water blue prepared with physiological saline solution in twice distilled water, and covered with a cover glass. FM examination or counting must begin during the first 10 minutes. Errors would naturally result again if the staphylococcal suspension were kept longer in the water blue because the copper-red plasma fluorescence of the dead cells would likewise become quenched.

The following details in testing this method are worth noting. Staphylococcal material from a 2-day-old culture was first immersed in the acridine orange solution. Centrifugation was immediately followed by a careful FM examination of the bacterial material without water blue. Only 7% of the cells exhibited a green plasma fluorescence due to the rather delicate membrane, while 93% exhibited red fluorescence mainly in the membranes, and the fluorescence of the plasma contents could not be easily distinguished in the majority of these cases. At the same time a specimen was prepared in water blue from the same stained bacterial suspension. The membrane fluorescence was immediately quenched. A count showed 76% of the cells with green fluorescing plasma and 24% with copper-red fluorescing plasma. Cultivation experiments confirmed the situation observed in the water blue, but directly contradicted the results of subsequent treatment without water blue. With and without the water blue all the cocci appeared red and no signs of growth could be seen in the culture.

It is evident from these experiments that the consecutive acridine orange-water blue method can be used to ascertain the vitality of staphylococci and that the underlying theoretical assumptions mentioned above are correct.

Cultivation experiments with Hottinger nutrient agar plates containing water blue showed that the dye does not injure staphylococci in the slightest. Neither the addition of 1:1000, 1:5000, or 1:10,000 water blue had any effect whatever, as compared with the control, on the growth of the staphylococci.

Streptococcus pyogenes humanus

The behavior of this form is similar to that of Staphylococcus aureus. The membranes are quite solid and the volume of the protoplasm is very small. Thus, these streptococci after immersion in 1:10,000 acridine orange prepared with physiological saline solution in twice distilled water also appear red (under low magnification) when living. A careful examination with the best lenses shows that it is mainly a matter here of red membrane fluorescence. Use of the consecutive acridine orange-water blue method (immersion in 1:5000 acridine orange prepared with physiological saline solution in twice distilled water) makes it possible to get rid of the membrane fluorescence in this object too so that only the green plasma fluorescence appears. Toxicity studies show that acridine orange does not cause any significant injury to the streptococci within the first 20 minutes.

Sarcina lutea

Staining; Sarcina lutea from a fresh culture with 1:10,000 acridine orange prepared with physiological saline solution in twice distilled water gives rise to the following distribution of the dye. The plasma of a few cells fluoresces green. Observations showed that it is a question of young dividing individuals. The bulk of the cells possess distinct solid, red fluorescing membranes through which the green protoplasts can be discerned. The plasma fluorescence seems to be completely quenched in many packets and cells owing to the strong membrane fluorescence. There are occasional swollen, solidly copper-red Sarcina cells. These cells tend to die. The behavior of Sarcina lutea is thus comparable to that of the staphylococci. Here too the very powerful membrane effect does not permit the use of the simple acridine orange dyeing technique to distinguish between living and dead cells.

The consecutive method involving 1:5000 acridine orange prepared with physiological saline solution in twice distilled water and 1:5000 water blue prepared with physiological saline solution in twice distilled water makes it possible to get rid of the membrane coloring, despite the persistence of the copper-red fluorescence, so that the vitality of Sarcina cells can be precisely determined under the FM. Comparative studies on living and artificially killed material carried out with the consecutive and cultivation methods confirmed the correctness of this conclusion. Quantitative analysis of Sarcina is difficult owing to the packet form.

Various Species of Bacilli

A great variety of bacilli isolated from soil as well as B. nombycis, B. mesentericus, B. mycoides, and B. subtilis were used to analyze acridine orange fluorochroming. These strongly gram-positive species were found to have a more or less pronounced membrane effect, although

they differ from one another in behavior. The large B. bombycis exhibits much less disturbance of plasma fluorescence due to red membrane fluorescence than do the smaller forms such as B. subtilis and B. mesentericus. If a suspension from a young culture (12-15 hours) is placed in 1:10,000 acridine orange prepared with tap water or physiological saline solution (tap water is much better for soil bacteria) and examined under the FM, the bulk of the bacilli will appear to have an intense, distinct, copper-red membrane coloring. In many forms, however, the membranes are so distinct that they can be recognized from their bright copper-red contours. The cell content of living bacilli then appears dark, as was the case with the gram-positive cocci. The cell content of dead bacilli examined closely under the FM have a solid red coloring. Many species, especially B. bombycis, have numerous short dividing bacilli which lack a solid membrane and consequently their plasma fluoresces pure green. Moreover, there are many transitional forms, depending on the species investigated, which exhibit both a red membrane fluorescence and a green translucent plasma fluorescence. All this indicates that the bacilli within a single culture are not physiologically equivalent and that bacilli of various ages or developmental stages may be present.

Use of the consecutive method (immersion in 1:10,000 acridine orange and examination in 1:1000 to 1:20,000) yielded the same results as in the gram-positive cocci described above. The red membrane fluorescence disappears immediately in the water blue while the green plasma fluorescence is no longer visible. There is no doubt that living and dead bacilli can be differentiated in water blue solutions in concentrations suited to the individual species. There are forms in which the membrane fluorescence is quenched so easily that a water blue concentration of 1:20,000 is right. The right concentration is determined by comparing living and dead material with the consecutive method and changing the water blue concentration. That concentration is the right one in which the protoplasm of the living cells fluoresces green while that of the dead cells fluoresces copper red within the first 10 minutes.

The behavior of sporulating bacilli is of particular interest. A FM examination of such material stained with 1:10,000 acridine orange shows that most of the endospores within the bacillus are prominent because of the weak dark green fluorescence of their protoplasm when living. The spore membranes are usually stained more or less reddish, thereby greatly weakening the green plasma fluorescence. Dead spores appear solidly shiny copper red. If the spore is already developed, the residual plasma of the bacillus is solidly copper red and therefore probably dead. The basic acridine orange method is suitable for differentiating between living and dead spores, although the water blue method can also be used. For this purpose the highest possible water blue concentrations are selected (1:1000 to 1:5000). Spore-containing material first dyed in 1:5000 acridine orange is immersed in 1:1000 to 1:5000 water blue and then examined under the FM. The water blue quenches the membrane fluorescence of the spores, but it cannot penetrate

into the interior of living or dead spores owing to its low degree of dispersion. Consequently, in these specimens the plasma of the living spores fluoresces green while that of the dead spores fluoresces a shiny copper red. Refinement of this technique is highly desirable for the study of the physiology of spore resistance.

In connection with the cytology of the larger species of bacilli, it should be mentioned that sometimes vitally stained material contains vacuoles and distinct yellowish-green fluorescing inclusion bodies in the protoplasm. I was unable to determine for sure whether these intensely stained bodies are vitally stained nucleoids. Here systematic studies should be carried out with the new vital staining methods.

Azotobacter chroococcum and Azotobacter beijerinckii
Strain 3 and Strain K₇*

Azotobacter belongs to the largest diplococci found in nature. It is further characterized by a solid mucous capsule. It was therefore of particular interest both to investigate the structure of the protoplast in vivo by means of acridine orange vital fluorochroming and to study the reaction of the gelatinous capsule to acridine orange. Since Azotobacter is a gram-positive coccal form the volume of whose protoplasm is in a very favorable ratio to the membrane, it was to be expected that Azotobacter would ensure an optically easier evaluation of the vital fluorochroming of the protoplasm than the much smaller gram-positive micrococci.

Azobacter lives in soil. Tests of various solvents for acridine orange (physiological saline solution, tap water, well water) showed that natural conditions are best approximated by using tap or well water. Vital staining with an acridine orange solution prepared with physiological saline solution proved to be highly toxic because the Azotobacter cells are not salt-resistant. This behavior was confirmed in all cases by both the counting and cultivation methods. FM examination of material stained with 1:10,000 acridine orange in tap water showed a sharp separation between the membrane formations and protoplasts. The large round to oval protoplasts of the living cells fluoresced a pure green and revealed a space latticelike distribution of the protoplasm in their interior. (Fig. 20). This latticelike structure is further emphasized by the fact that intensely yellow-green fluorescing granules can frequently be seen in the corners of the lattice. It is conceivable that these formations, which react to acridine orange like nuclear substances, are identical to nucleoids since they mostly occur in a definite number (one, two, seldom more). However, further study of the matter is necessary. The interstices between the lattice do not fluoresce and hence appear dark. This lattice structure cannot always be seen so clearly. In quite young cultures we found many rapidly dividing cells with densely structured protoplasm so that a lattice structure could be barely discerned. The membranes invariably fluoresce

*I obtained the Azotobacter strains through the kind cooperation of Prof. Ripper-Baldes in Göttingen, for which I thank him most sincerely.

copper red and in case of stronger development show gloeal-capsulelike stratification which is emphasized by unusually intense membrane fluorescence. The substance lying between the red fluorescing layers has a weak dark red color. There are, however, developmental stages of the Azotobacter cells which have only a simple, copper-red fluorescing membrane, and I was sometimes able to find cells with no distinct membrane at all. Due to the large size of the green fluorescing plasmatic body, the red fluorescing mucous capsules only rarely interfere with the FM appraisal of the plasma fluorescence.

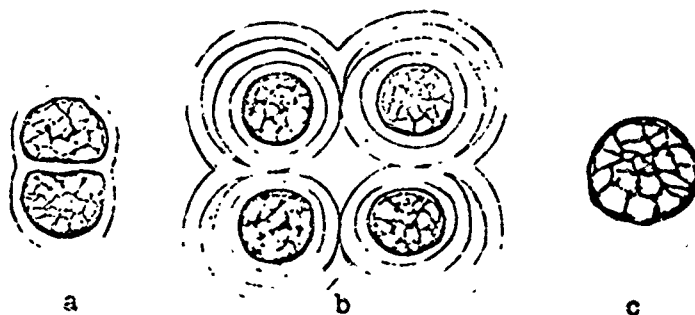


Fig. 20. Azotobacter chroococcum drawn from specimens vitally stained with acridine orange: (a) two-cell colonies; (b) four-cell colonies with beautiful gelatinous capsules; (c) a cell without a pronounced gelatinous membrane. Gelatinous membranes fluoresce red, the alveolar cytoplasm fluoresces green, containing 1-2 yellow-green compressions in the compartments.

In order to prove that such fluorochromed Azotobacter cells are viable and that the structural peculiarities observed in these cells also have a vital character, growth experiments were performed with vitally stained cells in microcultures. On the other hand, Azotobacter cells with copper-red fluorescing protoplasts were also observed in these microcultures. This made it relatively easy to demonstrate once again the precision of the acridine orange method as a means of differentiating between living and dead cells. The microcultures carried out on Rippel's agar clearly revealed that the cells whose protoplasts fluoresced green were capable of dividing, whereas the cells whose plasma fluoresced copper red remained undivided. It follows, then, that the acridine orange method is fully applicable to gram-positive Azotobacter cells and that the vital plasma structure described above is not an artifact.

It is also interesting to note that the addition of sodium chloride or potassium nitrate causes the gelatinous capsule of Azotobacter to

swell and liquefy. KNO_3 is best used in a 0.1% concentration. Unlike NaCl , it does not injure the protoplasts. A 1/150 molar phosphate buffer prepared from primary and secondary potassium phosphate (pH 6.5) is also recommended as a solvent for vital fluorochroming of Azotobacter cells.

Mycobacterium tuberculosis

The vital staining of tubercle bacilli is a matter of especial interest to medicine. For years I have been carefully testing acridine orange on both the human and avian types. The gram-positive nature of the tubercle bacilli seems to be related to the formation of a lipid-containing membrane layer and the very first experiments showed that M. tuberculosis is quite different from other gram-positive bacteria with respect to acridine orange staining. They react to the dye like gram-negative bacilli.

After immersion of the bacteria in a 1:10,000, 1:5000, or 1:1000 acridine orange solution in physiological saline, FM examination discloses excellent differentiation of the microorganisms. All living cells fluoresce green while dead cells fluoresce a bright copper red. Intermediate tones are regularly observed. Most of the cells survive, the percentage of dead bacteria normally being quite small. All the quantitative analyses showed that M. tuberculosis is completely insensitive either to the type of solvent or to acridine orange itself. Thus, there is no limitation on the results of using the dye on this highly resistant microorganism. It is also immaterial whether ordinary or twice distilled water is used. Moreover, since M. tuberculosis is likewise withstands drying, air-dried smears can be placed on slides and vitally fluorochromed.

Smears from a pure culture of Mycobacterium tuberculosis var. avium were placed on defatted slides and allowed to dry in the air. Part of the specimen was killed by heat fixation over a flame, while the other part was treated in an unfixed state. Both experimental series were dyed with 1:1000 acridine orange (tap water) for 5 minutes and then examined under the FM. Most of the bacilli in the unfixed specimens fluoresced green, whereas those in the heat-fixed specimens generally fluoresced copper red.

Bacilli from a pure culture were placed in a test tube containing 1:1000 acridine orange (physiological saline solution). The results of the toxicity test are shown in Fig. 21. The curves are virtually horizontal, i.e., neither the solvent nor the dye is capable of killing the bacteria gradually. This shows that M. tuberculosis is very well suited for the acridine orange method and that this dye will be the method of choice for future studies on the resistance physiology of tubercle bacilli.

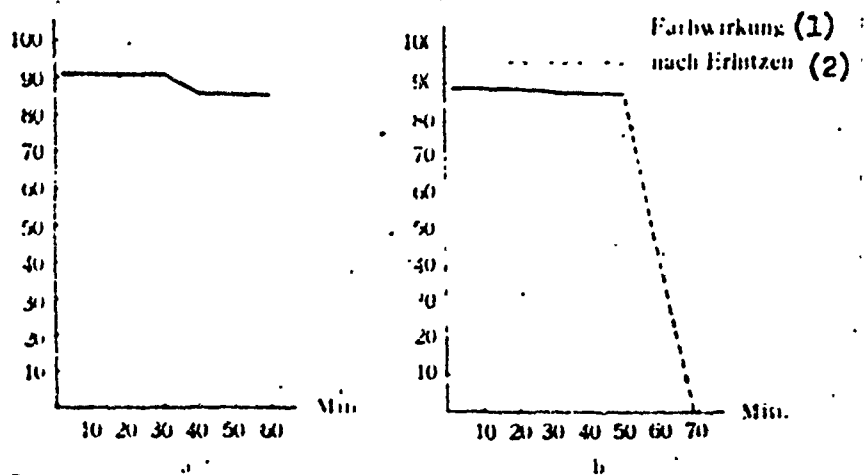


Fig. 21. *Mycobacterium tuberculosis* var. *avium* from a young culture suspended in 1:1000 acridine orange (dissolved in physiological saline solution). A count of the cells (every 10 minutes) whose protoplasm fluoresced green and thus were living showed no significant decrease in the number of living cells. Abscissa: duration of dyeing in minutes; ordinate: percentage of living tubercle bacilli counted at this time. (a) Series of experiments without subsequent heat fixation; (b) series of experiments with heat fixation after 60 minutes.

1 - dye action; 2 - after heating

These results completely coincide with earlier findings on the extraordinary resistance of tubercle bacilli.

Close examination of various old pure cultures vitally stained with acridine orange frequently revealed surprising differentiation in the interior of the bacilli, described in the literature in the form of numerous bodies and granules. I should like to point out here that such studies have become feasible with the help of vital acridine orange fluorochroming.

I have likewise tested in numerous experiments the cultivability of tubercle bacteria vitally stained with acridine orange. I found no difference between cultures on Petraghani nutrient media and unstained controls. Through the cooperation of my esteemed colleague Professor Wagner I was also able to determine whether vitally fluorochromed bacteria retain their virulence or not. Experiments on guinea pigs clearly showed that tubercle bacteria vitally stained with acridine orange remain completely virulent.

Living bacilli were found in air-dried sputum smears stained with acridine orange that were obtained from tubercular patients. However, this method is not absolutely reliable because other bacilli in the sputum are fluorochromed and the acridine orange method does not ensure clear differentiation of acid-resistant bacilli.

Observations on *Nosema bombycis* Nageli and *Nosema apis* Zander

The microsporidian *Nosema* is the causative agent of an economically important disease of silkworms and bees. Since Hilbrich (1942) made a detailed study of acridine orange vital fluorochroming of *Nosema* spores under the direction of Professor Miessner and myself, I may be allowed to add a summary of this work to my description of the bacterial forms.

The question of whether living and dead *Nosema* spores can be perfectly distinguished by the acridine orange method is of practical importance because cultivation experiments with this microsporidian are very difficult to carry out. Testing of the usefulness of the acridine orange method is therefore of practical importance in finding a suitable disinfectant for *Nosema* spores. Hilbrich clearly showed that living *Nosema bombycis* and *Nosema apis* spores stained with 1:5000 acridine orange in physiological saline solution exhibit distinct green fluorescence of the protoplasm while dead spores fluoresce only copper red. Hilbrich was able to confirm this finding by infection experiments. He used the acridine orange method to show that the compound Mycomor even in low concentrations can kill *Nosema* spores quickly and surely. A 1-2% NaOH solution killed the spores within one minute. Hilbrich concluded that the acridine orange method has made the cumbersome and somewhat unreliable infection experiments unnecessary in *Nosema* research.

CELL PHYSIOLOGY OF BACTERIA

Introduction

Bacteriology has been cultivated from a variety of standpoints in the course of the historical development of biology and medicine. It was, above all, the focal point of theoretical biological interest. The problems of spontaneous genesis were closely bound up with the history of bacteriology. When it became evident from Pasteur's and Koch's studies that bacteria play a prominent practical role in major technological processes as well as in human and veterinary medicine, bacteriology was increasingly abandoned by the botanists and this branch of science was left for physicians and industrial mycologists to develop. Bacteriology owes much to medicine for its extraordinary progress. However, it must be noted that neglect of the field by botanists and plant physiologists resulted in practical matters being

accorded more attention than theoretical questions. For example, the physiology of bacterial cells became largely ignored as far as protoplasm research was concerned.

A branch of botany called protoplasmatics has developed within the last 50 years. The aim of this discipline is to investigate the physical chemistry of the living cell, mainly in the higher plants. Bacterial cells were used for this purpose from time to time. It is also in the interest of medicine for the botanical side to be more fully represented in the solution of the problem in bacterial cells. Fluorescence microscopy has given renewed impetus to such studies within the past few years.

Use of the acridine orange method makes it possible to study microscopically the structure of living, vitally died bacterial cells and the course of the destruction of the protoplasts. Up to now physiological research has developed more in the direction of biochemistry and medicine with respect to pathogenicity. The modern FM technique, however, has made microscopic study of the physiology of bacterial cells possible. The following chapters describing some observations on the physiology of bacterial cells represent only the very beginning steps in this direction. I hope that they will contribute to the creation of an experimental protoplasmatics of the bacterial cell.

Plasmolysis

An essential criterion of the plant cell is its plasmolyzability. In a hypertonic medium so much water is lost from the vacuole by osmotic action that the protoplast separates from the membrane and contracts. This process is intimately related to the vitality of the protoplast. Plant cell physiology uses plasmolysis as an important aid. It is of value in determining variations in osmosis, viscosity, and permeability.

If the organization of the bacterial cell is similar to that of other plant cells, we would expect bacterial cells transferred to a hypertonic medium to exhibit the phenomenon of plasmolysis. The older literature indeed contains some positive references to the plasmolyzability of the bacterial cell. Fischer (1891) was the first to describe the process of plasmolysis in bacterial cells lying in a hypertonic medium. The protoplast separates from the membrane. It contracts and becomes round more or less in conformity to the laws of surface tension. In rod-shaped cells he was also able to observe the formation of several partial protoplasts. Fischer was unable to see plasmolysis *intra vitam*. He fixed and stained bacteria and observed plasmolytic contraction under the microscope. Savini (1909), Reichert (1919), Wohlfeil (1927), and Raichel (1928) completely confirmed Fischer's findings.

There are, to be sure, references in the literature which deny that bacterial cells are plasmolyzable (Gehrmann, 1901; Eisenberg, 1919). The reasons for this contrary view are that all bacteria are not equally plasmolyzable and that not all plasmolytics are capable of inducing bacterial plasmolysis. Fischer (1891, 1895, 1900, 1906) states that plasmolysis is easily achieved, in general, only in gram-positive bacteria. On the other hand, a more recent observer (Elo, 1937) reports on successful plasmolysis of gram-positive Bacillus subtilis in a 1 M grape sugar solution. A close relationship between plasmolyzability and gram-specificity, as repeatedly conjectured in the literature, therefore does not seem to exist. Elo (1937) and Höfler (1938) rightly assume that plasmolysis in gram-positive bacteria is difficult because the protoplast is more closely connected with the cell membrane than is the case with gram-negative forms.

Elo (1937) and Höfler (1938) were the first to evaluate, from the botanical standpoint, bacterial plasmolysis in problems involving permeability. Both authors, unlike Fischer, carried out their studies on living undyed material in a bright field. Fischer himself (1891) emphasized that intravital staining of membranes and protoplasts would greatly facilitate the observation of bacterial plasmolysis.

Acridine orange vital fluorochroming satisfies Fischer's wish completely. I therefore urged my co-worker Raffelt (1944) to carefully investigate plasmolysis in gram-negative bacteria with this method. Bacterium coli proved to be the most suitable object for this work. Since the protoplast of a living bacterial cell fluoresces green while the delicate membrane fluoresces red, the process of plasmolytic contraction of the protoplast can be accurately traced intra vitam. Raffelt studied the morphology of bacterial plasmolysis in detail. His main objective was to compare the plasmolyzability of coli capable of being stained various fluorescent colors with acridine orange. He could thus clearly test the acridine method of differentiating between living and dead bacterial cells by investigating plasmolyzability.

Raffelt's technique was simple. Coli bacteria were introduced into a test tube containing 1:5000 acridine orange (with physiological saline solution). A loopful of the suspension was then transferred to a drop of the plasmolytic, after which the specimen was covered with a cover glass and examined under the FM. Counterstaining in the plasmolytic proved to be helpful in enhancing the visibility of the membranes of the cells undergoing plasmolysis. The prestained material is transferred to the plasmolytic and then mixed with acridine orange (1:5000 to 1:10,000). It is also possible to transfer the unstained bacterial material directly to the plasmolytic already mixed with acridine orange. The FM examination must be carried out with the finest FM, immersion lenses, and strongest light available.

If coli bacteria are placed in 1 mole of grape sugar, the bacilli with red fluorescing plasma exhibit no changes in the plasmolytic. The bacilli whose protoplasts fluoresce yellow or yellow-red likewise

remain unchanged. This category of bacterial cells is thus not plasmolyzable and is to be regarded as dead or injured. The coli bacilli with red fluorescing plasma, on the other hand, exhibit pronounced morphological changes. Superficial examination shows that the green fluorescing bacilli are greatly shrunken and irregular in contours. It is only after careful examination of counterstained material that one realizes it is a question of true plasmolysis. The detachment of the green fluorescing protoplasmatic body from the very delicate, red fluorescing membrane can be clearly seen. In general, the protoplast is rather uniformly elevated in convex form from both poles (Fig. 22) or along the longitudinal wall of the bacilli (Fig. 23).



Fig. 22. Diagram of a coli bacterium plasmolyzed with 1 mole of grape sugar and vitally fluorochromed with acridine orange. Perfect convex plasmolysis (from Raffelt, 1944).

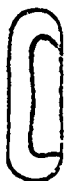


Fig. 23. Coli bacterium plasmolyzed with 1 mole of grape sugar and vitally fluorochromed. Lateral elevation of the protoplast. Pronounced negative plasmolysis site on a longitudinal wall (from Raffelt, 1944).

Plasmolysis in 1 mole of grape sugar cannot be tolerated very long by B. coli without injury. The strong hypertonic jump results after 5-10 minutes in gradual death of the plasmolyzed bacilli. When examined in an acridine-orange-containing plasmolytic, a red fluorescence appears in the protoplasm as the microorganisms die off.

B. coli cannot be plasmolyzed in 1 mole of glycerin. It is fair to assume that glycerin is permeated so quickly that plasmolysis is impossible. However, plasmolysis takes place much more quickly in 1 mole of urea than it does in grape sugar. but gradual deplasmolysis soon sets in because urea is fairly easily permeated. Raffelt's comparative observations showed that urea is permeated much more slowly than glycerin. According to Collander's formulation (1937), B. coli can be considered a glycerin type.

Raffelt induced very good plasmolysis in 1 mole of NaCl. Contraction of the green fluorescing protoplast is so strong that often no more than a small green dot can be seen.

I conducted similar intravital plasmolysis studies on vitally fluorochromed Azotobacter (especially strain G, Göttinger), with 0.1-0.2 mole of grape sugar solution or 2% NaCl solution serving as the plasmolytic. The low osmotic value in these diplococcal cells is surprising. Even a 0.1 M grape sugar solution is clearly hypertonic. Fig. 24 presents a series of drawings which show the plasmolysis forms observed. Regular plasmolysis forms produce concave elevations along the periphery or on one side, although convex forms often appear. Meanwhile elevation of the protoplast from the solid membrane can be clearly seen. In this object too I demonstrated once again that only Azotobacter cells with green fluorescing protoplasm are plasmolyzable, whereas cells with yellow or copper-red fluorescing protoplasm do not undergo plasmolysis.

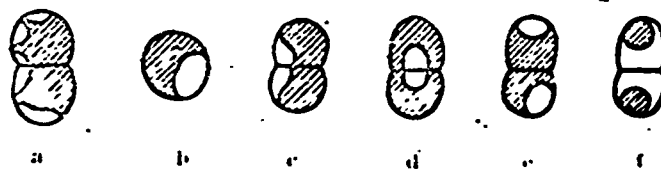


Fig. 24. Azotobacter diplococci in 0.2 mole of grape sugar fluorochromed and plasmolyzed. a - f - the various plasmolysis forms observed. a - concave elevations in various places; b - a concave elevation; c - lateral plasmolysis; d - pronounced positive plasmolysis site on the transverse wall; e - positive plasmolysis sites on the poles; f - negative plasmolysis sites on the poles.

These tentative studies on bacterial plasmolysis clearly show that the acridine orange method is suitable for further systematic research on permeability in living bacterial cells, a phenomenon that also possesses medical significance.

Effects of the Medium

An important matter for bacteriologists is the effects of the medium on the maintenance of bacterial viability. My co-worker Raffelt (1944) studied the effects of a change of medium on some bacteria using both the acridine orange and the cultivation methods. Gram-negative forms like Bacterium fluorescens were studied quantitatively in the course of prolonged suspension in twice distilled water, ordinary distilled water, and physiological saline solution. Fig. 25 shows the results of a 20-day experiment. B. fluorescens was suspended in one of the media and allowed to stand in the incubator at a constant

temperature. The AO method was used for statistical determination of living and dead bacilli. At the same time parallel cultures were established in order to compare the viability of the bacteria. B. fluorescens survived a considerable length of time in the physiological saline solution, but in twice distilled water a high percentage of the cells (40%) died during the first stage of the experiment due perhaps to injury caused by hypertonicity. Ordinary distilled water injured the bacteria even sooner because of its content of oligodynamic metal traces.

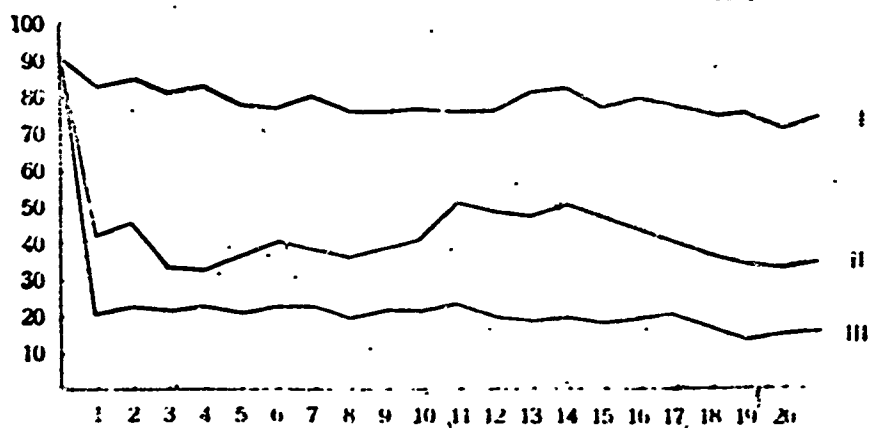


Fig. 25. The effect of a physiological saline solution (I), twice distilled water (II), and ordinary distilled water (III) on a suspension from a fresh B. fluorescens culture. Abscissa: duration of immersion in the medium in days; ordinate: percentage of living cells in the suspensions determined with the acridine orange method (from Raffelt, 1944).

In B. coli, too, Raffelt found a large, experimentally reproducible difference between the effects of twice and ordinary distilled water. If twice distilled water is first brought into contact with silver or copper, it becomes as toxic as ordinary distilled water (Fig. 26).

When B. fluorescens, B. coli, and B. proteus are suddenly transferred to a 1 M sugar solution, 50% of the bacteria die during the first stage of an experiment. The surviving microorganisms then are resistant for a long time. Raffelt observed the same phenomenon after transfer to 1 mole of glycerin, urea, KNO_3 , or CaCl_2 . As to be expected, the appearance of some differences of individual nature often appear among the various bacterial forms in such studies.

The knowledge gained to date with the acridine orange technique on the effect of the medium is already sufficient to show that the individual bacterial forms vary in degree of resistance so that a single formula for the medium cannot be devised. Our experience is that

physiological saline solution prepared with twice distilled water is undoubtedly the least toxic to the pathogenic forms. For soil bacteria, on the other hand, the physiological saline solution is too concentrated and, moreover, the NaCl is toxic. Tap water here is a better medium by far. In the case of saprophytic bacteria, the most suitable medium for a maximally sparing suspension of living microorganisms has to be first determined experimentally in each individual case by means of the acridine orange method.

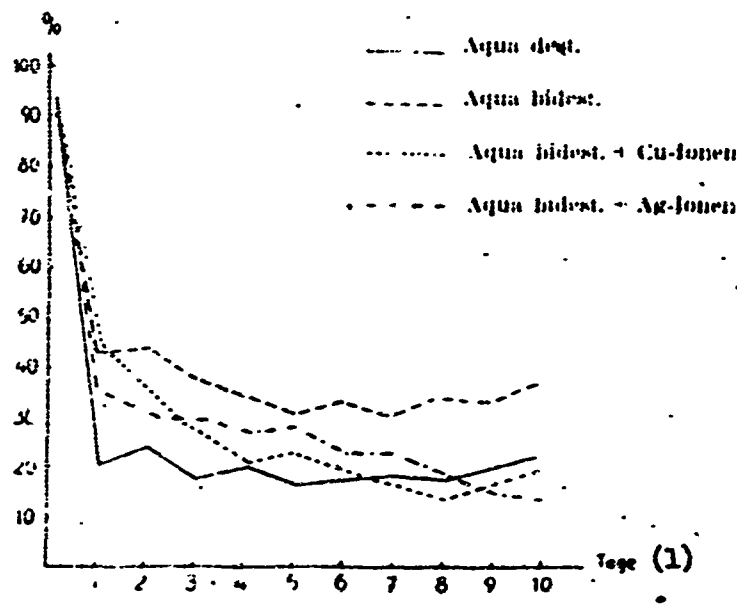


Fig. 26. The effect of ordinary distilled water, twice distilled water, and twice distilled water with Cu and Ag ions added on the viability of a B. coli suspension. Abscissa: duration of action in days; ordinate: percentage of living bacilli determined with the acridine orange method (from Raffelt, 1944).

1 - days

Resistance Physiology of Bacteria

Vital fluorochroming of bacteria with acridine orange is important in enlarging our knowledge of the resistance physiology of these microorganisms. On the assumption that the bacterial forms under study are stainable, direct FM diagnosis of plasmatic injury or death offers a completely new methodological possibility for investigating the effect of extreme external conditions on the vitality of the bacterial cell. Therefore, I should like to report briefly the most important results of work done in my institute.

Schneider (1944) used the acridine orange method to study the effects of methyl and ethyl alcohol. The bacterial material was treated with different concentrations of methyl or ethyl alcohol for 5 minutes. After fluorochroming with acridine orange, the dead and living cells were counted. The FM results were checked by cultivation experiments. All the pathogenic bacteria, with the exception of Mycobacterium tuberculosis, exhibited a complete coincidence between the appearance of red fluorescing protoplasm in the acridine orange experiments and the loss of viability in the cultivation experiments. Thus, the acridine orange method made it possible to determine with precision the death of gram-negative bacterial protoplasm caused by alcohol. The toxic effect of methyl alcohol on B. coli is shown in Fig. 27. The alcohol percentages are plotted on the abscissa, the percentages of living bacilli on the ordinate. The duration of action was 5 minutes in all the experiments. It is apparent from the curve that a substantial number of bacilli survived in solutions with less than 40% methyl alcohol content, that many were quickly injured when the content was between 40 and 60%, and that the entire population died when the content was over 60%. Ethyl alcohol produced the same effect.

The results of Schneider's studies on Mycobacterium tuberculosis are worth special mention. Material was taken from a good culture of M. tuberculosis var. avium and placed in various alcohol concentrations for an hour. This was followed by fluorochroming with acridine orange and counting of the living and dead bacilli in the usual way. (Fig. 28). Unlike the case with B. coli and other types, ethyl alcohol does not seem to affect the viability of the tubercle bacteria. The percentage of cells with green fluorescing protoplasm remains within the same statistical boundaries. Parallel experiments with material pretreated with alcohol produced the surprising result that no further growth could be achieved on Petraghani nutrient media.

Since the observation has both theoretical and practical implications, Prof. K. Wagener, Director of the Hygienic Institute of the Veterinary College, Hannover, and I repeated Schneider's experiments and confirmed them by animal experiments. The tubercle bacteria pretreated with alcohol are no longer cultivable and experiments on guinea pigs clearly showed that such bacteria are no longer able to infect the

animals. The following theories are offered to account for the abnormal behavior of the tubercle bacteria:

(1) The tubercle bacteria pretreated with alcohol are indeed still living; and, consequently, their protoplasts fluoresce green after acridine orange staining, but their viability and virulence are destroyed by alcohol. The acridine orange method reveals the structural features characteristic of living protoplasm, but it cannot indicate inhibition of growth.

(2) The protoplasm of tubercle bacteria pretreated with alcohol is indeed dead, but the acridine orange method fails to work in this case.

The decision between the two possibilities was reached in the following way. Heating tubercle bacteria pretreated with alcohol at 100° for several minutes causes the protoplasm stained with acridine orange to fluoresce red. This observation plus the fact that all the other bacterial forms studied after treatment with alcohol had copper-red fluorescein protoplasts which died after acridine orange staining convinced me that the first possibility is the more likely. The protoplasm of tubercle bacteria is not so drastically altered by alcohol that it must be regarded as dead. It is fair to assume, therefore, that alcohol irreversibly halts only the growth capacity of the bacteria.

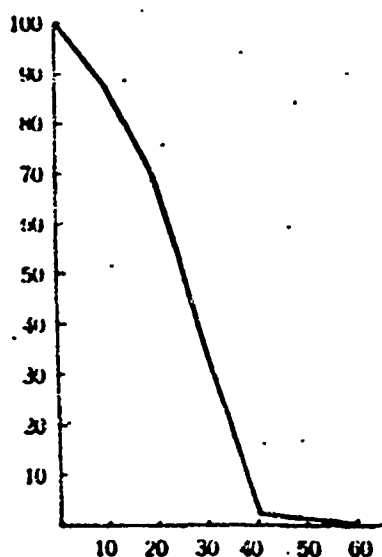


Fig. 27. Toxic effect of different concentrations of methyl alcohol solutions on B. coli. Duration of action of each concentration 5 minutes. Abscissa: alcohol percentages; ordinate: percentage of living coli bacilli counted with the help of the acridine orange method (from Schneider, 1944).

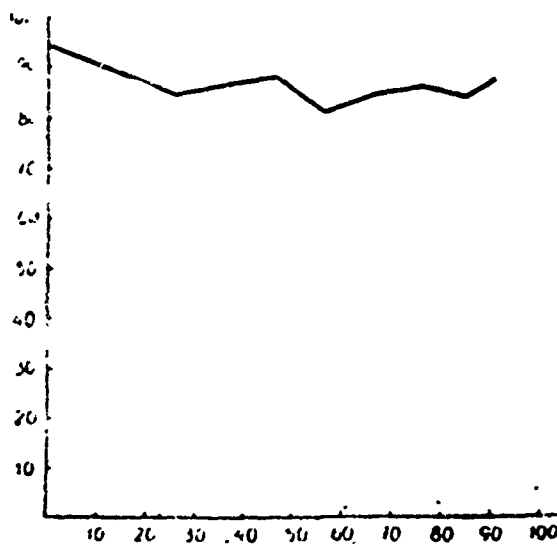


Fig. 28. Toxic effect of different concentrations of ethyl alcohol solutions on M. tuberculosis var. avium. Duration of action 1 hour. Abscissa: alcohol percentages; ordinate: percentage of living tubercle bacteria counted with the help of the acridine orange method (from Schneider, 1944).

Hefner (1944) reports on the use of the acridine orange method to study resistance to drying in some bacterial types, i.e., Bacterium fluorescens, Bacterium coli, and Bacterium pullorum. The bacterial material was taken from 12-hour-old cultures with a loop and suspended at the rate of 2 loopful per 3 cc of desiccating medium. The following were used as media: distilled water, physiological saline solution (0.85%), horse meat broth, horse serum, 1.5% gelatin in distilled water, and 1.5% gelatin in physiological saline solution. Smears of the bacterial suspensions were placed on slides at a room temperature of 22° C for drying. Resistance to the drying was investigated by means of the acridine orange method and cultivation experiments. Air-dried smears were stained from time to time in an acridine orange solution (1:5000) prepared in physiological saline solution, after which the living and dead germs were counted under the FM. Hefner found the FM results to be completely consistent with the cultivation data. Whereas the fresh starting cultures contained approximately 98-100% living bacteria, Hefner noted after 20 minutes' drying in distilled water a decrease of around 20% in the number of bacteria with green fluorescing protoplasm. During the next four days the percentage of living bacteria gradually decreased to zero. The desiccated gram-negative bacilli behaved similarly in physiological saline solution. The gram-resistant bacilli dried in meat-broth smears proved to be much

more resistant. Some 50% were living after 20 minutes' drying, but the number dropped to less than 1% after six more days. The surviving bacteria even after 20 days' drying were treated as living both from the EM and cultivation standpoints. Bacteria dried in serum also showed a considerable increase in resistance. The relations were even more favorable in the case of B. coli dried in gelatin.

According to Hefner, the chief injury to the bacteria occurs directly after air drying. Resistance to desiccation is very low in water and in saline solution, but it increases markedly in a colloidal medium. With respect to the physiology of the bacterial cell, it is particularly interesting to note that even under the most favorable drying conditions, e.g., in serum or gelatin, only a few cells survive. There must be a definite physiological difference between the individuals in a given bacterial population. According to Hefner, the rate of desiccation is determinative of the degree of injury to a bacterial population.

It is difficult to decide whether the desiccated bacteria are already dead when stained or the injury following remoistening results from too rapid absorption of water. Comparative experiments indicate that the latter is the more probable cause of the principal injury. Rupture (plasmolysis) of the bacterial cells occurs regularly in hypotonic media. Hefner succeeded in directly observing such plasmolysis in vitally stained material when remoistened. The cell wall ruptures at one pole or longitudinal wall and small, sharply contoured balls of protoplasm often emerge from the bacterial body.

According to my findings on Mycobacterium tuberculosis, this bacillus proved to be unusual in respect to resistance to drying. Living bacilli were suspended in tap water or distilled water and smears from the suspension were placed on fat-free slides. After they dried, both the acridine orange and cultivation methods revealed that none of the bacilli died. M. tuberculosis proved to be highly resistant to drying in all my experiments.

Local Degenerative Phenomena in Bacterial Protoplasts

The acridine orange method is a sensitive indicator of protoplasmatic structure. Even localized necrotic changes in the plasmatic body of a cell are clearly revealed under the microscope. In 1940 I was able to demonstrate with this method the local necrosis of Allium cepa epidermic cells after localized puncture of living protoplasts with a microneedle. The puncture area fluoresced copper red in an acridine orange bath while the rest of the protoplasmatic body fluoresced green.

Not only local necrosis in metaphyte cells can be detected in this way, but also spreading of the structural changes caused by death of the protoplasm. This is also true of rod-shaped bacterial cells and spirilla, as was observed in the course of comprehensive bacteriological

investigations conducted in my institute. Both spirilla and rod-shaped bacteria regularly show a locus minoris resistentiae at both poles of the cell. The operation of injurious factors such as desiccation, hypertonicity, oligodynamic and toxic effects gives rise to highly localized injury at either or both poles. Fig. 29 shows schematically such poles of necrosis in rod-shaped bacilli. The protoplasmatic body regularly undergoes strictly limited necrosis at either or both poles. These parts of the poles fluoresce a shiny copper red after acridine orange staining while the center of the protoplasts still shows the green fluorescence of living bacterial protoplasts. It is worth noting that this necrosis causes a dumbbell-shaped enlargement of the necrotic area due, perhaps, to a swelling effect. This is the phenomenon generally observed in gram-negative bacilli that on dying they become wider as a result of swelling.

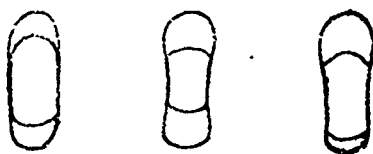


Fig. 29. Schematic representation of polar degeneration of bacterial protoplasts. The black places in the protoplasm fluoresce copper red after treatment with acridine orange while the white places fluoresce green (after Hefner, 1944).

The partial necrosis that occurs in Spirillum is particularly impressive. When spirilla treated with acridine orange are injured, the protoplast does not die uniformly all at once. There is regular polar degeneration. This polar degeneration spreads gradually forward toward the center of the spirilla. Such spirilla are still motile, but their power of movement is limited. Eventually the flagella beat so slowly that the power of movement is virtually nil. It ceases completely as soon as the protoplast fluoresces shiny copper red.

Polar degeneration of bacterial protoplasts warrants further intensive scientific study. It must be determined, above all, whether bacteria in this stage are still capable of growth. Polar degeneration of bacteria is also of particular interest from the standpoint of the hit theory of radiation effects. Additional investigations are needed to elucidate the problem of the death of bacterial cells.

FLUORESCENCE-OPTICAL ANALYSIS OF SOIL FOR MICROORGANISMS

Introduction

Bacteria, fungi, algae, and lower animals, as inhabitants of the soil, are of the greatest importance in all the transformations that take place there. The physical, chemical, and mineral characteristics of the soil as well as the climate are influenced, above all, by the activity of the soil microorganisms. Hence, all the methods used to analyze the content of microorganisms are of great theoretical and practical value for soil science and for agricultural practice. We know that agriculturally good soils are also rich in microorganisms, whereas agriculturally poor or useless soils are very poor in microorganisms. Stable manure is good for plants not only because it contains chemical nutrients, but also because it is richly supplied with microorganisms. All measures to improve the soil should also help to promote the living conditions of the microorganisms. This is basic knowledge to which no specialist today can shut his eyes and it explains why agricultural soil microbiology has developed into a very important, independent science.

Soil microbiologists have two methods of analyzing soil: (1) plate cultures to grow microorganisms, (2) direct microscopic analysis of the microorganisms in soil.

Add. 1. The plate culture method is used most often because it presents the fewest difficulties. Diluted soil samples are inoculated onto synthetic nutrient media of various kinds (chemical composition, pH, oxygen supply) and soon after incubation the living germs appear on the plates in the form of colonies. The advantage of this method is that the microorganisms can be quickly isolated in pure cultures and are thus relatively easy to describe systematically. The plate count technique was devised for quantitative analysis. Plates are inoculated with a certain amount of diluted soil suspension and after a while the growing microorganisms are counted. The plate count and plate culture methods are now very popular and they provide extensive factual material. Numerous bacterial forms can be isolated from soil and counts yield values of 100-300 million per gram of soil. It must be noted, however, that the plate method has several major shortcomings. It is quite obvious that not all living bacteria inoculated onto a given nutrient medium will find favorable growth conditions. The pH of the medium, chemical composition, and oxygen supply are limiting factors in every plate culture experiment. We can safely count on the fact that all the bacteria or other microorganisms present in soil will never be encompassed in a plate experiment. The resultant values will always turn out to be too low.

It is a well known fact that the shape and size of the bacterial cell will frequently change greatly in relation to the substrate conditions.

Although autochthonous bacteria can grow on a synthetic medium, previous experience suggests that such microorganisms will differ in shape and size from inoculated autochthonous forms. There is no compelling reason, to assume, therefore, that the forms appearing in synthetic cultures grow the same way in the soil. The autochthonous state of the soil bacteria is likely to remain unknown when the plate culture technique is used.

Add. 2. All these shortcomings can be compensated if, simultaneously with the plate culture method, direct microscopic analysis of the autochthonous soil bacteria is feasible. There has been no lack of efforts to make such studies directly on stained soil smears. Conn (1918, 1929), for example, tried to stain soil smears with erythrosin in order to examine bacteria in their autochthonous form in the soil. Conn also succeeded in differentiating larger bacterial forms in such smears by staining them, although the similar coloring of the soil particles and the bacteria causes too many difficulties in observation to permit the approach to be considered an acceptable method in soil microbiology. Winogradsky (1925, 1928, 1929) introduced the slide method. He sank slides into the ground, preferably near plant roots, and removed them after a while. The bacteria which had grown in the autochthonous state adhered to the slides in a thin layer and were stainable. But under these conditions the tiniest soil particles are stained like bacteria so that great care and experience are required to evaluate such specimens. Moreover, both living and dead bacteria are involved and they cannot be distinguished by this method. This raises doubts especially regarding the counts made by Conn and by Winogradsky. Attempts to observe soil bacteria in the disorderly mass of unstained soil particles failed.

In summary, it can be stated that thus far no solution has been found to the problem of carrying out qualitative and quantitative analyses of living bacteria in their autochthonous state in the soil directly under the microscope. The foregoing discussion amply explains the theoretical and practical necessity for further systematic progress in this field.

Fluoroscopic Analysis of Soil Bacteria Using the Acridine Orange Fluorochroming Method

In 1941 I undertook for the first time to apply the method to the study of autochthonous soil bacteria. It became immediately apparent that acridine orange fluorochroming is the best way of making a useful, direct microscopic analysis of the soil microflora. These preliminary experiments clearly showed that humus-covered soil particles can store the acridine orange cations so intensively owing to their strong negative charge (acid nature) that after adequate exposure of the soil suspension to the dye all the soil flakes and crumbs and mineral particles fluoresce dull red. The living bacteria resting on the soil particles stand out quite distinctly in their green fluorescence against the dull

copper-red background. Dead bacteria are stained solid copper red and they fluoresce much more intensely than does the dull red background of the soil particles. Bacteria floating about freely in the soil suspension can, of course, be observed without any difficulty. Thus, fluorochroming of soil suspensions with acridine orange seems to be highly suitable for direct microscopic analysis of soil. The following are some of the advantages over other staining techniques:

- (1) It permits far-reaching microscopic differentiation between living and dead soil bacteria;
- (2) The soil particles fluoresce a different color from that of the living bacteria adhering to them;
- (3) Consequently, morphological analysis of the living autochthonous bacteria is possible and, in addition, the topographic distribution of the microorganisms in the soil can be studied;
- (4) Since vital fluorochroming causes minimum injury, vitally stained bacteria can be cultured further.

With my colleague Dr. Christine Rouschal I carried out a systematic study of acridine orange fluorochroming of soil suspensions after the preliminary experiments were concluded. The early results have already been briefly reported (Rouschal and Strugger, 1943). I also published another report (1937). That such a new method, developed in a few years under frequently difficult working conditions, could not yet produce satisfactory results goes without saying. We would therefore greatly appreciate the help of others in solving the problems that have arisen.

Acridine Orange Fluorochroming of Soil

Acridine orange fluorochroming of soil suspensions must in technical respects be systematically determined for each soil since the capacity for adsorbing the dye naturally differs from soil to soil. Sandy soils with a relatively low adsorption capacity are best fluorochromed with lower concentrations of the dye than are suitable for humus-rich soils, which have a high adsorption capacity. The excess dye remaining in the suspension fluid should not be too great, for otherwise FM observation of the cover glass specimens prepared from the soil suspension would be influenced by the intense acridine fluorescence of the suspension fluid. Nor should the concentration of acridine orange be too low because adsorptive saturation of the soil particle surfaces would not be achieved. This would result in very poor FM contrasting of the living bacteria against the background of the soil particles because humus substances fluoresce green rather than red if inadequately

* I thank my friend Baron Dr. Stephan von Thyssen-Bornemisza for help in this work.

stained. The correct dye concentrations are determined by preparing 1:500, 1:1000, 1:2000, 1:3000, and 1:5000 acridine orange solutions in tap or well water. Freshly obtained soil samples are promptly fluorochromed and examined. The samples are passed through as fine a sieve as possible. Several 1 g portions (fresh weight) are measured off from the sifted soil, poured into test tubes, mixed with 100 cc of each concentration of the dye, and vigorously shaken. The soil suspension can be considered fluorochromed after 5-10 minutes, a conservative figure, for experience has clearly shown that soil samples can be thoroughly stained in a much shorter time. In preparing a microscopic specimen, very small drops are taken from the suspension, placed on a slide, and covered with the thinnest cover glass available. Since one must work with immersion objectives at a short distance, the presence of large grains of sand in the specimen must be prevented at all costs. Examination in liquid paraffin produces very beautiful pictures, especially for FM judgment of the bacterial forms present. For this purpose the fluorochromed soil suspension is strongly centrifuged off. As much of the supernatant dye solution as possible is poured off so that the stained silt remains in the sediment. A small drop of liquid paraffin is placed on a slide and, using a platinum loop, a trace of the stained earth is rubbed into the drop of paraffin, which is again covered with a cover glass. The advantage of using liquid paraffin is that fluorescence of the suspension fluid is completely eliminated and the contrast effect of the pictures is greatly enhanced. The liquid paraffin method is not suitable, of course, for counting.

These specimens are observed under as powerful a blue-light FM as possible; an instrument with weak light is useless. We recommend the large Zeiss FM or Reichert FM and auxiliary apparatus with a mercury-vapor high-pressure lamp as a light source. Only the best immersion lenses should be used. In our work we used either a Zeiss 60 apochromatic objective, N.A. 1.4 or a Zeiss 120 apochromatic objective, N.A. 1.2. The Reichert "fluorescence-free 100 x" objective proved to be the best of all. Fluorescence-free oil or liquid paraffin was used as immersion oil. The soil preparation is first examined with rather weak dry lenses to find the most favorable places, not too thick and not too thin. The immersion lenses are then adjusted and the investigation starts.

This procedure is applied to the same soil sample stained with various concentrations of acridine orange. In the case of normal garden soil, the 1:500 concentration is in most cases unsuitable for fluorochroming soil suspensions because excess dye remains in the fluid. While the coloring of the soil particles is of course good and bacteria can be seen, nevertheless too much detail is lost because of the surrounding intensely fluorescing dye solution. Concentrations between 1:1000 and 1:2000 are in general the best. The remaining excess dye is within tolerable limits so that the fluorescence of the sample is optically satisfactory. Concentrations between 1:3000 and 1:5000 often produce too weakly stained specimens. The humus elements then fluoresce

a dirty yellowish red rather than a bright red, weakening the desirable contrast with the bacterial cells.

Therefore, the absolute rule for fluorochroming soil samples is that the dyeing must in each case be done in graduated acridine orange concentrations. For a given soil that concentration is right in which the background (i.e., the suspension fluid) fluoresces unobtrusively while the soil particles fluoresce bright red. Once the most favorable concentration is found the work can proceed without interruption.

Qualitative Results to Date with Acridine Orange Fluorochroming of Soils

Soil suspensions fluorochromed with acridine orange show the following pictures. Most soil particles, if they consist of humus or mineral particles covered with humus, fluoresce a distinct copper red or brownish red. Only a few particles, evidently those without a pronounced humus adsorption layer, fluoresce a weak yellowish red or greenish. On the soil particles coccal and rod-shaped structures fluorescing a distinct green lie anchored to the humus layers. In addition, good soils quite often contain zooglee-like accumulations of green fluorescing cocci or bacilli. Copper-red, rod-shaped structures and structures resembling cocci are also found on the soil particles, but they are less common. They stand out clearly against the background because they fluoresce a more intense copper red than do the soil particles. Most of the green fluorescing structures thought to be bacteria are extraordinarily small. The resolving power of the best objectives is insufficient to permit them to be seen clearly. The autochthonous bacteria range from 0.2 to 1 μ in size. Larger cocci and bacilli 1 to 5 μ in size also occur, but they are much fewer than the smaller forms. The bacterial nature of these larger structures is clearly shown by the observable cell organization. The smaller cocci, on the other hand, are much more difficult to judge. It is often a question whether these tiny green fluorescing dots are really living bacteria or not. The objection might be raised that aggregates of soil colloid particles can store acridine orange only in a green fluorescence color. The bacterial nature of these structures is confirmed by the fact that equally small bacteria whose nature is beyond doubt appear in zooglae in massive numbers in a gelatinous matrix. The following experiments were performed to clarify this critical matter.

Soil obtained from the 5 cm layer in the garden of the Veterinary College was placed in 4 large test tubes (5 g of soil in each) and plugged with cotton wads.

- (1) Sample at room temperature - the control.
- (2) Sample mixed with 10 cc of 35% formalin.

(3) Sample with 20 cc of tap water boiled at intervals over a period of 4 days for a total of 9 hours.

(4) Sample with nothing added, sterilized in stages in a drying oven at 160° over a period of 4 days for a total of 9 hours.

The dyeing was carried out with acridine orange in tap water (1:1000). FM examination yielded the following results:

Sample 1. The soil proved to be rich in bacteria. Almost every particle contained one or more coccal and rod-shaped structures at the limit of microscopic visibility. They fluoresced green, which we found to be typical of living bacteria. Also numerous larger cocci, bacilli, and occasional zoogloas of undoubted bacterial nature could be seen on the soil particles. Only a few green bacteria were detected floating freely in the suspension fluid.

Sample 2. 1 cc of the sample treated with formalin was pipetted off. The earth was allowed to settle and the formalin was poured off. 10 cc of the dye solution was added. The formalin had no effect on the dyeing. However, the sample showed a markedly different picture from control. No fluorescing bacilli or structures resembling cocci could be seen on the soil particles stained copper red. The smallest bacteria-like forms now shone copper red. This was also true of the larger forms and of those found lying free. There was no doubt that the formalin killed the protoplasm of these very small cocci and bacilli and large bacterial forms, causing them to fluoresce copper red. Hence, transfer to malt and Hottinger agar failed to result in any bacterial growth.

Sample 3. In the boiled sample the pictures were more or less like those in sample 2. The bulk of the bacteria-like green fluorescing pictures disappeared and in their place were red fluorescing structures. There were also a few tiny green bacilli. Parallel plate cultures showed some islands of bacteria.

Sample 4. The sample oven-dried at 160° likewise showed no green bacteria and inoculated culture plates remained sterile.

This series of experiments clarified a fundamental problem. The structures capable of being fluorochromed green by acridine orange in untreated soil and which are so small that their bacterial nature is not immediately recognized can be changed by destructive measures into a state characteristic of dead protoplasm. They undoubtedly represent autochthonous bacteria comparable to the rickettsias in size. A simulation of tiny soil bacteria by green fluorescing lifeless structures is perhaps rendered impossible by these very carefully executed analyses. No such bacteria-like particles exist in the soil. This proves that by using the acridine orange method one can see perfectly bacteria of all microscopically still resolvable sizes in autochthonous state in the soil.

Observations employing the acridine orange method were collected for the purpose of describing autochthonous bacteria in a great variety of soils. It is evident that the smallest bacilli and cocci which lie at the limit of microscopic visibility constitute the bulk of the bacterial flora. Larger bacilli and cocci appear often, to be sure, but they are much less numerous than the smallest forms. Most of the latter do not live freely in the soil water, but they adhere to the surface of microscopically small soil particles. They rest either free on flakey humus crumbs or on minute mineral particles. These are covered with a layer of humus adsorbate in which the bacteria live. Zooglea-like accumulations are to be seen more often than not on the surface of soil particles in soils with a very rich bacterial content. These gelatinous zoogleas are either spherical or irregularly flat. In soils that are very rich in bacteria, the surfaces of the particles are absolutely covered with bacteria, which also fill every corner and fold (cf. Plate III, 1, 2, 3, 4). Along with these small forms are green diplococci, chains of bacilli, and yeast cells, likewise anchored to the surfaces of the soil particles (Fig. 30). Only a small percentage of the bacteria can be seen floating about freely.

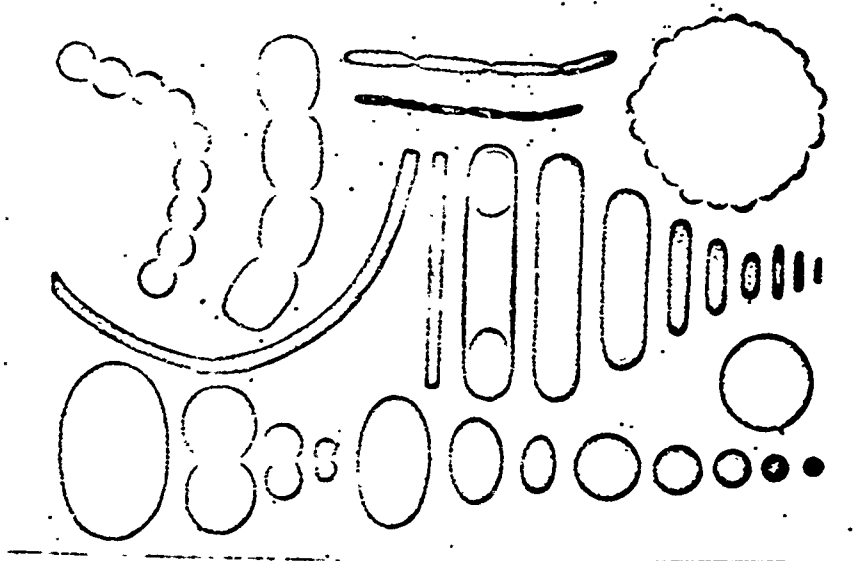


Fig. 30. Types of autochthonous soil bacteria observed. The proportions are full scale.

The distribution of bacteria in the soil is not uniform. Alongside humus particles thickly crowded with bacteria are completely bacteria-free particles. This stands to reason, for the living conditions are not equally favorable for the microorganisms on all the soil particles. For example, humus-deficient quartz particles may have very few bacteria and in very sandy soils the presence of bacteria-free particles is in fact the rule. The following excerpt from a study of a garden soil illustrates the autochthonous bacterial forms occurring in the soil.

Fresh garden soil from the top layer was stained with 1:1000 acridine orange in tap water for 10 minutes. After being vigorously shaken, the suspensions were allowed to stand until the large soil particles settled. The fine suspensions were the first to be examined. Noted were: small to tiny bacilli, extraordinarily slender; thicker bacilli; tiny cocci; diplococci; thicker oval bacilli; long, very slender bacilli; bacilli united with pealike zooglea; micrococci united with pealike zooglea; zooglea of presumably the smallest bacilli lying at the limit of visibility; mycobacterium-like, branched bacilli; densely packed large cocci.

Besides fine suspensions, specimens of coarse sediment were also investigated. On the whole, the shapes were the same. Large, thread-like chains of bacilli and long chains of streptococci were occasionally seen. Large masses of zooglea also appeared in the coarse sediment.

The following may be said of the size of the bacterial forms found in the soil. Very small cocci and bacilli $\frac{1}{2}$ to 1μ in size definitely predominate. Only relatively few forms are larger. The large forms clearly occur more often in garden soils fertilized with stable manure or in meadow soils than in forest soils. This suggests that the larger bacterial forms were probably introduced into the soil secondarily.

Soil yeasts were common. They occurred in their characteristic range of sizes.

Motile bacterial forms cannot be seen in the soil. It must be emphasized, however, that fully motile bacterial forms can be grown in microcultures prepared with soil samples. It is conceivable, therefore, that motile forms are also present in the soil. Perhaps the power of locomotion of free floating cells is impaired by staining.

Azotobacter-like diplococci were frequently seen in soil samples, but they were completely lacking in gelatinous membranes.

Sporulating bacilli were rendered visible by acridine orange fluorochroming. The spores fluoresced mostly green. Isolated bacterial spores cannot be immediately made prominent or detected in acridine orange fluorochromed soil. For this purpose I worked out a special fluorochroming technique described below (p. 139).

Dead bacteria are undoubtedly more difficult to detect in soil than living bacteria. Their fluorescence color contrasts only slightly with the substrate. But with some practice even dead bacteria can be recognized. Besides, they are much less important for bacteriological observation than living microorganisms. We were always astonished at the relatively few dead bacteria present in the soil. When samples of deep-lying soil were analyzed, the number of dead bacteria increased surprisingly. This is possibly due to the death of specific anaerobes on exposure to air.

Soil fungi are easily detected in acridine orange fluorochromed soil samples. They are found first in the coarser sediments of the soil suspension and they can be detected with weak or medium dry lenses. Living fungal hyphae fluoresce either uniformly green or have green fluorescing protoplasm together with intensely red fluorescing vacuoles in their interior. Fungus spores are also present in the soil.

Fluoroscopic Analysis for Chlorophyll-Containing Microorganisms

Examination of soil suspended in pure water under a bright-field microscope barely reveals the presence of a few diatoms, filamentous algae, chlorellas, desmids, or cyanophyceans in the confused mass of soil particles. It is absolutely important to judge the content of chlorophyllaceous microorganisms even under low magnification. The FM offers significant advantages in this respect. The chromatophores of these microorganisms exhibit very intense blood-red fluorescence of the chlorophyll. They can be very clearly recognized by this primary fluorescence in a soil suspension prepared with tap water. Under low magnification chlorophyllaceous cells can be quickly and surely identified by their intense red fluorescence. Fluorescence of chlorophyll is generally pronounced only in living cells. In dead cells the chlorophyll is usually destroyed or transformed and thus it no longer fluoresces distinctly. The suggested fluoroscopic analysis, therefore, has the further advantage of generally indicating the presence of living chlorophyllaceous microorganisms. Diatoms, blue algae, green algae, protonemata, and other forms show a blood-red fluorescence of their chromatophores. Algal forms cannot, of course, be identified solely through fluorescence. However, once an algal cell is detected by its fluorescence, it can easily be recognized again and identified by changing over to bright-field illumination. Only the chromatophores have an intense red primary fluorescence. This is not true of the soil particles.

Our experience with algae is consistent with that of other investigators. They occur mostly in the top layers of the soil and rarely penetrate beyond the 10 cm layer. Soil fungi and soil algae are decidedly less numerous than bacteria.

Fluorescence-Microscopic Observation of Bacterial Spores in the Soil

Since acridine orange fluorochroming cannot be used for clear evaluation of the content of bacterial spores in a soil sample, it seemed necessary to develop a dyeing technique to provide sharp contrast for the spores in the soil. Diachroming methods are completely useless in solving this problem. Here again fluorochroming steps into the breach. The procedure for elective fluorochroming of bacterial spores with brilliant sulfoflavin and coriphosphine (p. 36) can be advantageously adapted to elective visualization of these spores. It makes all the spores fluoresce yellow-green, all the nonsporulating bacteria red. And it facilitates clear differentiation between spores and cocci. If a smear is fluorochromed first with brilliant sulfoflavin and then with coriphosphine, only the spores fluoresce yellow-green while the nonsporulating bacteria and the soil particles store coriphosphine and hence fluoresce red. Thus, the yellow-green fluorescing bacterial spores contrast very sharply with their background. This spore staining in the soil is not vital staining. It can be carried out only on heat-fixed smears.

Brilliant Sulfoflavin-Coriphosphine Staining for Elective Visualization of Bacterial Spores in the Soil

- (1) Preparation of a soil suspension in tap water.
- (2) Smears prepared from a soil suspension on well-defatted slides and dried.
- (3) Good heat fixation.
- (4) Dyeing with a 1:400 brilliant sulfoflavin FF solution in distilled water plus 0.5% phenolum liquefactum; heating for one minute over a pilot burner until the formation of vapor.
- (5) Good rinsing in distilled water.
- (6) 10-40 seconds' counterstaining with 1:1000 coriphosphine in distilled water.
- (7) Washing out with distilled water.
- (8) Air dried, covered with a drop of liquid paraffin and a cover glass.

The specimens thus prepared are examined under a powerful blue-light FM. The bacterial spores show either a solid or peripheral intense yellow-green fluorescence while the soil particles and non-sporulating bacteria fluoresce red. The spores are $\frac{1}{2}$ to $2\frac{1}{2}\mu$ in size and are round to oval in shape. They lie separately or in clusters, isolated or on bits of earth. The spores are conveniently observed

in these specimens and they can also be counted. Spores were observed in every soil. However, the spore content varies considerably. For example, it is larger in cultivated garden soils than in forest soils.

Fluorescence-Microscopic Counting of Living Bacteria in the Soil

Until now bacterial counts in the soil have been made either with the plate method or, in some cases, in diachromed smears. The errors made with the plate method were discussed in the introduction to this chapter. They are so big that the resultant values are undoubtedly much too low. Conn (1918, 1929) and Winogradsky (1925, 1928, 1929) obtained higher values in diachromed smears. Also many bacteria escaped these authors since the soil particles often store the dye like the bacteria resting on them and the bacteria, therefore, can no longer be discerned.

Acridine orange fluorochroming, on the other hand, enables us to improve the counting procedure considerably. All living bacteria, including those on earth crumbs (the bulk of them) can be seen perfectly under the FM. Even the smallest forms, which are so characteristic of the autochthonous soil bacteria, can be counted. It is in the nature of things that no method of counting bacteria is faultless, and I am of the opinion that the FM counting of acridine orange fluorochromed soil samples proposed here is theoretically incapable of further improvement. However, even this method can produce errors which are technically not avoidable:

(1) With direct counting only the bacteria resting on one side of the soil particles can be clearly seen; those on the reverse side cannot be seen.

(2) Zoogless cannot be counted; they can only be estimated.

(3) Statistical errors are unavoidable.

In view of these possible sources of errors, the following method of counting living bacteria was used. 1 g of finely sifted soil was taken from a large sample, suspended in 10 cc of 1:1000 to 1:5000 acridine orange (tested thoroughly), and vigorously shaken. The acridine orange concentrations must be very carefully tested since it is important to observe the smallest bacteria without interference. 1 cc is pipetted from the thoroughly shaken suspension as quickly as possible, while avoiding decantation, and diluted tenfold with pure, sterile tapwater. The counting was carried out with a Zeiss counting chamber, which consists of an extrathin slide and no screen separation. A chamber 20 μ deep proved to be best. We were able, however, to work effectively with a 50 μ deep chamber. But a layer 100 μ thick was too thick. The counting chamber is covered with a completely flat cover glass. Observation takes place with the large Zeiss FM using the Zeiss counting eyepiece

in Ehrlich's modification with the adjustable square diaphragm in position 3. The Zeiss apochromatic 60, N.A.1.4 is used as the objective. Several specimens were prepared from a single soil sample and 10 random fields of view were counted in each specimen. Each square field of view was carefully examined for the following bacterial deposits, the numerical values being noted separately for each: (1) the bacteria resting on the soil particles; (2) the bacteria resting on the edge of the soil particles; (3) the bacteria floating about freely in the suspension fluid.

Since just as many bacteria, statistically speaking, are on top of the particles as are on the bottom and since only the former can be counted, a third of the bacteria resting on the particles was added to the final average, a figure surely too small rather than too high.

The zoogeleas were noteworthy. These heaps of tiny bacteria embedded in gelatin cannot be counted. However, the number of zoogeleas can be indicated and an estimate made from the size of the individual bacteria and from the size of the aggregation. Such estimates are always somewhat understated in order to be on the safe side.

The number of zoogeleas in 10 counted fields of view produces the zoogelea value, which invariably rises in parallel with increase in the bacterial content of a soil. Thus, the zoogelea value has great practical significance. In average soils the zoogelea value with 100-fold thinning is about 1. In soils with a greater abundance of bacteria the value may rise to 4.5.

In counting it is also important to determine the final value from the maximum number of individual values because the bacteria are very unevenly distributed in the various soils. Hence, the soil must be well mixed before weighing and, for statistical levelling of the values, shaken before the counting is started. We generally used 5 different specimens of the same soil sample and counted 10 fields of view in each so that the final average was based on 50 fields of view. A complete count of 100 or more fields did not change the values significantly.

The following investigation is described in detail to illustrate the kind of data recorded and the method of calculation.

Soil from the 2-5 cm layer in the garden of the Veterinary College of Hannover. 1 g fresh weight weighed off to determine the dry weight and 1 g fresh weight prepared for staining.
1 g fresh weight = 0.678 g dry weight.

1 g fresh weight of weighed off soil dyed in 10 cc of 1:4000 acridine orange. Filled to 100 cc with tap water after 5 minutes.

The soil is thus thinned 100-fold. Preparation of specimen after careful shaking with a counting chamber 0.05 mm deep. Ehrlich's counting eyepiece in position 3.

Table 29

(1) 1. Präparat	1	2	3	4	5	6	7	8	9	10	(5) Mittel	Zoogl.	(6) Mittelwert der Summe
(2) Aufsatz	16	14	8	50	—	12	50	6	14	—	17,2	1	
(3) Rand	3	—	2	—	—	7	—	—	—	—	1,2		
(4) frei	—	—	1	—	2	—	—	—	—	1	0,4		19,8
(1) 2. Präparat													
(2) Aufsatz	16	14	21	15	16	6	16	12	50	31	19,5	2	
(3) Rand	—	2	2	—	—	—	—	—	—	—	0,4		
(4) frei	—	1	2	—	1	1	2	—	—	—	0,7		20,6
(1) 3. Präparat													
(2) Aufsatz	22	15	18	50	6	30	50	22	17	4	23,4	3	
(3) Rand	—	1	6	—	—	—	—	—	—	—	0,7		
(4) frei	—	2	3	6	—	—	3	1	—	3	1,8		25,0
(1) 4. Präparat													
(2) Aufsatz	18	26	27	15	21	17	40	17	12	16	20,4	1	
(3) Rand	—	—	—	—	—	—	—	—	—	1	0,1		
(4) frei	2	—	3	—	—	1	—	—	2	3	1,1		22,1
(1) 5. Präparat													
(2) Aufsatz	15	21	50	24	13	21	15	21	20	—	18,7	1	
(3) Rand	—	—	—	—	—	—	—	2	—	—	0,2		
(4) frei	1	6	—	—	8	—	1	—	—	1	1,7		20,6
(7) Gesamtmittelwert 21,6													
(8) Gesamtmittelwert der aufsitzenden Bakterien 19,94													
(9) Gesamtmittelwert der an den Rändern sitzenden Bakterien 0,52													
(10) Gesamtmittelwert der freien Bakterien 1,14													

1 - Specimen; 2 - resting; 3 - edge; 4 - free; 5 - average;
6 - mean of sums; 7 - total mean value 21.6; 8 - total mean value of bacteria resting on soil particles 19.94 = 92.31%; 9 - total mean value of bacteria on the edges 0.52 = 2.45%; 10 - total mean value of free bacteria 1.14 = 5.27%.

9 zoogleas on 5 specimens yielded a zooglea value of 1.8 = number of zoogleas in 10 fields of view with 100-fold thinning of the soil. Correction for the bottom of the soil particle = total mean 21.6 plus 1/2 of the bacteria resting on them 6.64 = 28.24. This figure represents the final mean value.

Calibration of the counting eyepiece: with position 3 and apochromatic lens 60 one side of the square field of view is 135 μ . Consequently,

the volume of the soil suspension diluted 100-fold with a chamber depth of 50μ is $135\mu^2 \cdot 50\mu = 911,250\mu^3 = 0.00091125 \text{ mm}^3$.

This is the volume of the square counted through

0.00091125 mm^3 contain 28.24 bacteria on the average.

1 mm^3 of soil suspension thus contains 30,990.3 bacteria.

After thinning the soil 1 mm^3 of soil suspension contains 1/100,000 g of fresh soil.

1/100,000 g of soil contains 30,990.3 bacteria. Therefore, 1 g of soil contains $30,990.3 \cdot 100,000 = 3,099,030,000$ bacteria. This figure applies to the fresh weight of the soil. The dry weight is computed from $\frac{3,099,030,000}{678} \cdot 1000 = 4,570,840,708$, i.e., 1 g dry

weight soil contains 4,570,840,707 living bacteria.

The zooglea value is 1.8 per 10 fields of view. Therefore, 1 field contains 0.18 zooglea. With a careful and surely too low an estimate 1 zooglea contains about 100 individual bacteria. Therefore, 1 field contains an average of 18 bacteria. The final mean value of 26.24 per field is thus raised to 46.24.

When this correction is introduced, the bacterial content per gram fresh weight and per gram dry weight comes to 5,074,350,000 and 7,484,292,030, respectively.

These bacterial counts set very high optical requirements. They can be made only with the powerful Zeiss FM under optimum conditions (best arrangement of the light source, oil drops between condenser and specimen, thinnest cover glasses, no interfering secondary fluorescence of the suspension fluid).

Bacterial spores are not included in these counts. Only non-sporulating living bacteria are considered.

Table 30 summarizes the results of counts of living bacteria made in a variety of soil samples.

Table 30

(1) Bodenart	(2) Tiefe der Zählkammer in mm	(3) Wasseranteil in % Feldherde	(4) Gesamtzahl der abgetöteten Bakterien pro Gramm Substrat	(5) Wasseranteil in % Feldherde	(6) Gesamtzahl der abgetöteten Bakterien pro Gramm Substrat	(7) Wasseranteil in % Feldherde
(8) Gartenerde Oberfl.-Probe	0,02	—	11,2	—	1,21.000.000	—
(8) Gartenerde Oberfl.-Probe	0,02	11,5 %	11,6	1,60.000.000	1,21.000.000	—
(8) Gartenerde Oberfl.-Probe	0,06	12,9 %	5,4	4,34.000.000	1,76.000.000	1,6
(9) Gartenerde Oberfl.-Probe wie oben, aber 24 h bei 37°C	0,06	14,7 %	14,1	2,012.000.000	1,92.000.000	0,3
(10) Gartenerde Oberfl.-Probe wie oben aber 24 h mit 11,9 durchtränkt	0,06	0,2 %	21,6	4,81.000.000	1,72.000.000	0,9
(11) Bestir Wiesens- boden Oberfl.-Probe	0,05	30,0 %	42,4	8,640.000.000	6,945.000.000	4,5
(12) Heiden- Sandboden Oberfl.-Probe	0,05	9,5 %	8,9	1,039.000.000	941.000.000	—

1 - soil type; 2 - depth of counting chamber in mm; 3 - water content per g of fresh soil; 4 - total mean of counted bacteria per field of view; 5 - rounded bacterial count in relation to dry weight; 6 - rounded bacterial count in relation to fresh weight; 7 - statistical value of the number of zoogloea per 10 fields of view; 8 - garden soil - surface sample; 9 - garden soil - surface sample as above but kept 24 hours at 37°C; 10 - garden soil - surface sample as above but soaked 24 hours in H₂O; 11 - best meadow soil - surface sample; 12 - heath-sandy soil - surface sample

It is evident from this table that the values per gram of fresh soil range from one-half to six billion. The poorest soils were the sandy heath soils. The largest masses of bacteria were in compost soil and in a bog soil in Vesbeck an der Leine.

Of interest is a rough calculation of the volume ratio between the living bacterial masses and the dead soil particles. Assuming for 1 g of soil a volume of 1 cc and for 1 bacterium a volume of $1\mu^3$, the bacterial mass in the soils richest in bacteria constitutes 1/125th part of the soil mass, while that in the soils poorest in bacteria constitutes 1/2000th part, i.e., at best 8 cubic meters of bacterial mass and at worst $\frac{1}{2}$ cubic meter of bacterial mass per 1000 cubic meters of soil. Thus, 1000 cubic meters of soil contain on the average 3-4 cubic meters of living bacterial mass.

The vertical distribution of soil bacteria was also of interest. We found a good example of this in Northwest Germany - in a very fertile bog-meadow soil in Vesbeck an der Leine on a large pasture belonging to the farmer Heinrich Peter. The investigation was carried out in the spring of 1945, i.e., at a time of the year when soil life can be assumed to be especially rich and luxuriant.

Description of the vegetation: pasture meadow evenly covered with moss, Alopecurus pratensis predominating, Avena elatior, Phleum pratense, Poa pratensis, Trifolium repens, Ranunculus acer, Rumex acer, Rumex acetosella, Bellis perennis, some moss.

Soil profile:

- 10 cm humous loam, very friable, dense grass roots, dark-brown-gray.
- 20 cm light gray loamy soil, root horizon.
- 35 cm gray loam of light clayey character with numerous roots.
- 45-50 cm discontinuance of root horizon proper, clay with iron rust spots.
- 60-145 cm blue-gray tone with numerous rust spots and angular, hardened rusty brown to black iron hydroxide particles; very delicate living roots penetrate to this depth through worm passages.
- 165 cm blue tone with many rust spots and iron inclusions, living roots in worm passages.
- 180 cm pure blue-gray tone.
- 190 cm sandy layer.
- 195 cm ground water, roots reaching ground water through worm passages.

Corresponding qualitative description of the bacterial content of this profile:

- 1 cm maximum number of bacteria, countless zoogloas, bacteria of all kinds, mostly the smallest bacteria.
- 10 cm very rich bacterial content, great abundance of forms.
- 20 cm very large number of small cocci and bacilli, also larger forms.
- 35 cm very rich in bacteria, numerous zoogloas.
- 45-50 cm zoogloas extend only to this depth, ceasing more or less with the main root horizon, great richness of bacteria.
- 60-110 cm significant decrease in bacterial content; many bacteria appear copper red and thus dead. Anaerobic? Larger forms quite inconspicuous.
- 110-165 cm solitary living tiny cocci and bacilli; dead forms common.
- 180 cm very few slender cocci and bacilli; dead bacilli present.
- 190 cm bacterial content very meager, relatively many dead, smallest bacterial forms inconspicuous, medium-sized bacilli predominant.

It is clear from the above that bacterial growth is greatest between the 2 and 10 cm layers. This depth is richest not only in bacteria, but in algae and fungi. It also has many zoogloas. From 10 cm downward to the end of the root horizon (ca. $\frac{1}{2}$ meter) the bacterial content decreases and there are no more zoogloas. From the $\frac{1}{2}$ m layer down the number of living bacteria constantly decreases. With the end of the root zone the decrease proceeds very rapidly, although a few bacteria can be found down to the ground water.

Investigation of such profiles invariably shows that the number of bacteria increases greatly near roots. The rhizosphere of individual deep-lying roots is extremely rich in these microorganisms.

It is evident from our studies that two factors are of major importance in determining the bacterial content of soil - humus and moisture. The humus content can be easily calculated with the acridine orange method. Since humus particles fluoresce a solid copper red and mineral components covered with a layer of humus adsorbate acquire red fluorescence, the degree of red fluorescence (when viewed under low magnification) is an obvious criterion for estimating the humus content. A strong correlation has been shown to exist between

the humus content and the number of bacteria. All soil samples with a high humus content also have an abundance of bacteria. Sandy soils, however, are poor both in humus and in bacteria. Special studies will be needed to determine whether this parallel holds true for bog soils.

The second factor responsible for the bacterial content of soil is water. We observed in garden soil following a prolonged drought a doubling of the bacterial content two days after a rainfall. Laboratory experiments yielded the same results. Soil was allowed to dry out for a day at 37° while part of the same soil was soaked and kept covered at room temperature. The dry samples had about 2 billion bacteria per gram compared with the moistened samples which had about 4 billion per gram. Zoogloas too are less common in dry soils than in moist ones. Zoogloas and an increased bacterial content are found within a day in dry soils that are moistened.

The temperature is a relatively minor factor. Our experiments extending over a period of years failed to show any significant change in microbial life in relation to the time of year. Although microbial life was decidedly richer in the summer at optimum temperatures, no significant decrease in activity was noted in the winter, even after prolonged frosts.

Finally, as to a possible relationship between the bacterial content and the pH, we unfortunately still have no precise information. The matter requires special investigation. It is our impression that within wide limits the pH value does not exert any profound effect on the quantity of bacteria in the soil.

Suggestions for Microbiological Assays of Soil Samples with the Fluorescence-Microscopic Method

The acridine orange method of fluorochroming soil permits visualization of the autochthonous bacterial flora directly under the FM. Since the content of living soil bacteria can be thus determined, FM examination of soil samples stained with acridine orange will come to be the method of choice in questions pertaining to soil evaluation. The quantitative method of counting or the qualitative method of estimating can be used for bacteriological soil evaluations. The counting procedure described in the previous section is laborious and feasible only with the best apparatus. I believe that I am right in maintaining that such counting can perhaps be dispensed with for practical purposes in favor of an estimate of the autochthonous bacteria based simply on inspection. Such estimates could be prepared quickly and on a large scale in agricultural institutes. In view of the practical importance of such microbiological assays, I have proposed on the basis of personal experience a five-fold classification of soils (applied after analysis of 1 g of fresh soil stained with 10 cc of acridine orange):

Class I. Soils with the richest bacterial content. Several to many zoogloas can be seen in each field of view on the humus layer of the soil particles. Alongside is a large population of tiny cocci and bacilli. Also larger bacterial forms like cocci, diplococci, streptococci, and chains of bacilli are frequent. These soils are easily evaluated. There is no technical difficulty in finding the numerous bacteria and zoogloas.

Class II. The bacterial zoogloas are scattered about in the fields of view. However, some effort is required to find them. The zooglea value per 10 fields is generally 1. Small individual bacteria rest in large numbers on the soil particles while larger cocci and bacilli are much less prominent.

Class III. A characteristic of these soils is that the zoogloas cannot be found routinely. The zooglea value drops below 1. The number of individual small bacterial forms resting on the soil particles is significantly reduced. However, each particle has 1-10 living bacterial cells adhering to it. The large bacterial forms are rare.

Class IV. Zoogloas are completely absent. Only a few bacteria are found on the soil particles. However, the distribution of individual bacteria on the particles is still quite normal.

Class V. No zoogloas can be observed. Most of the soil particles are completely bacteria-free. Only a few living bacteria can be detected on isolated soil particles.

The proposed classification can be evaluated quite easily and surely refined with increasing practice. The proposal is not intended to be definitive. Rather, it is designed to show that on the basis of the experience gained to date, the microbiological aspect of soil quality can be determined without laborious counting.

The Problem of Autochthonous Bacterial Flora in the Soil

Because up to now specific insight into autochthonous soil bacteria was impossible for technical reasons, the introduction of acridine orange fluorochroming and FM examination of soil confronted soil microbiologists with brand new facts. It has been shown above all that bacteria in soil possess a different autochthonous form from that in plate cultures. Thus far we have never succeeded in positively identifying bacterial forms in soil with soil bacteria known from plate cultures. The autochthonous bacteria growing in soil are unusually small, whereas most of those grown on plates are huge. Obviously, the living conditions prevailing in soil are so different from those on plates that very different growth forms are necessarily involved. Soil bacteriologists will have to carry out systematic microcultivation experiments with stained bacteria to identify the autochthonous forms with those appearing on culture plates.

We started in a preliminary way such experiments on vitally stained material. From a soil suspension stained with acridine orange we isolated tiny soil particles on which individual autochthonous soil bacteria rested. These particles were placed in hanging drops in a nutrient solution containing acridine orange in a concentration of 1:10,000. The vitally stained bacteria began to divide and we observed the development of 3-7 μ bacilli from autochthonous forms about 1 μ in size due to division and luxuriant growth.

It is evident from these initial experiments that the large bacterial forms appearing in plate cultures are identical with the very small bacterial cells observable in soil by direct FM examination.

A new field is opening up here for soil microbiology. It is now possible to approach the problem of taxonomy of autochthonous soil bacteria. The method also provides new opportunities for practical agriculture. The effects of drought, precipitation, mineral fertilizers, and type of cultivation on soil bacteria can now be systematically studied using a direct method.

Bibliography

- Albach, W. 1927. "Über vitale Kern- und Protoplasmafärbung pflanzlicher Zellen (Vital Staining of Plant Cell Nuclei and Protoplasm). Z. wiss. Mikr. u. mikr. Techn. 44, 333.
- 1926. Zellenphysiologische Untersuchungen über vitale Protoplasmafärbung (Cell Physiological Studies on Vital Staining of Protoplasm). Protoplasma 5, 412.
- Arloing, Policard and Langeran. 1925. Beobachtung an verschiedenen Mikrobenkulturen unter der Wirkung der Strahlen der Quecksilberbogenlampe und Filtration durch Wood-Filter (Observations on Various Microbial Cultures Exposed to the Rays of a Mercury-Vapor Lamp and Filtration Through Wood Filter). Compt. rend. soc. biol. Paris 92, 261.
- Bachmann, M. and L. Finke. 1939. "Über die fluoreszenzmikroskopische Darstellung von Tuberkelbazillen im Gewebe (Fluorescence-Microscopic Visualization of Tubercle Bacilli in Tissue Culture). Dtsch. med. Wschr. 65, 1474.
- Badian, J. 1937. Sur la cytologie des levures. Bull. int. Acad. Polon. Sé. B. Sci. Nat. 61.
- Brandt, K. M. 1941. Physiologische Chemie und Cytologie der Presshefe (Physiological Chemistry and Cytology of Pressed Yeast). Protoplasma 36, 77.
- Bucherer, H. 1943. Experimentelle Untersuchungen über die Fluoreszenzfärbung toter und lebender Bakterien nach Strugger (Experimental Studies on Fluorescence Staining of Dead and Living Bacteria with Strugger's Method). Zbl. f. Bakt. II. Abt. 106, 81.
- Bukatsch, F. 1940. Die fluoreszenzmikroskopische Darstellung von Lebensvorgängen in der Pflanze, insbesondere der Zell- und Kernteilung (Fluorescence-Microscopic Visualization of Vital Activity in Plants, Especially Cell and Nuclear Division). Z. f. d. ges. Naturwiss. Heft 3/4, 90.
- 1941. Einige Anwendungsgebiete der Fluoreszenzmikroskopie. Ibid., Heft 9/10, 268.
- Bukatsch, F. and M. Haitinger. 1940. Beiträge zur fluoreszenzmikroskopischen Darstellung des Zellinhaltes, insbesondere des Cytoplasmas und des Zellkernes (Contribution to the Fluorescence-Microscopic Visualization of the Cell Contents, Especially the Cytoplasm and Nucleus). Protoplasma 34, 515.

Casper, H. and K. Brandt. 1940. Nukleotidumsatz und Wachstum bei *Neurospora crassa* (Nucleotide Decomposition and Growth in Pressed Yeast). *Protoplasma* 35, 507.

Clauberg, K. F. 1939. "Über die Verwendbarkeit des Fluoreszenzmikroskopes in der bakteriologischen Praxis, zugleich über die Frage des Vorkommens virusartiger Mikroorganismen in menschlichem Krebsgewebe (Usefulness of the Fluorescence Microscope in Bacteriological Practice and the Question of Virus-like Microorganisms Occurring in Human Cancer Tissue). *Klin. Wschr.* 18, 632.

Collander, R. 1937. "The Permeability of Plant Protoplasts to Non-Electrolytes." *Transact. of the Faraday Soc.* 190, vol. 33, 8.

Conn, H. J. 1918. "The Microscopical Study of Bacteria and Fungi in Soil." *N. Y. Agr. Exp. Stat. Geneva, Techn. Bull.* 64.

— 1929. "Use of the Microscope in Studying the Activities of Bacteria in Soil." *Jour. Bact.* 17, 399.

Dabelstein, H. 1939. Das Fluoreszenzmikroskop in der laufenden Tuberkulosedagnostik (The Fluorescence Microscope in Diagnosis of Tuberculosis). *Zbl. Bakt. I. Abt.* 143, 242.

Danckwortt, P. 1940. Die Lumineszenzanalyse im filtrierten U.-V.-Licht (Luminescence Analysis in Filtered UV Light). *Akad. Verlagsges. Leipzig*, 4th ed.

Déhéré, Ch. 1933. Nachweis der biologisch wichtigen Körper durch Fluoreszenz und Fluoreszenzspektren (Detection of Biologically Important Substances by Fluorescence and Fluorescence Spectra). *Abderhalden, Handb. d. biol. Arbeitsmeth. Abt. II, Teil 3*, 1.

— 1937. Die Fluoreszenz in der Biochemie. Monographie biol. Probleme (Fluorescence in Biochemistry. Monograph on Biological Problems). Paris, Les Presses Univ. de France.

— 1938. La fluorescence en microbiologie. *Schweiz med. Wschr.* 948.

Denzler, B. 1942. Beiträge zur Analyse der Fluorochromierung von Bakterien (Contribution to the Analysis of the Fluorochroming of Bacteria). *Diss. Tierärztl. Hochschule Hannover*.

Didion, H. 1939. Über den fluoreszenzmikroskopischen Nachweis von Tuberkelbakterien (Fluorescence-Microscopic Detection of Tubercle Bacteria). *Klin. Wschr.* 15, 1315.

Döring, H. 1935. Versuche über die Aufnahme fluoreszierender Stoffe in lebende Pflanzenzellen (Experiments on the Uptake of Fluorescing Substances by Living Plant Cells). *Ber. der Deutschen Botanischen Gesellsch.* 53, 415.

C
Drawert, H. 1937. Untersuchungen "über die pH-Abhängigkeit der Plastidenfärbung mit Säurefuchsin und Toluidinblau in fixierten pflanzlichen Zellen (Studies on the pH Dependence of Plastid Dyeing with Acid Fuchsin and Toluidine Blue in Fixed Plant Cells). Flora 131, 341.

— 1937. Das Verhalten der einzelnen Zellbestandteile fixierter pflanzlicher Gewebe gegen saure und basische Farbstoffe bei verschiedener Wasserstoffionenkonzentration (Reaction of Individual Cell Constituents of Fixed Plant Tissue to Acid and Basic Dyes with Various Hydrogen Ion Concentrations). Flora 132, 91.

— 1939. Zur Frage der Stoffaufnahme durch die lebende pflanzliche Zelle. I. Versuche mit Rhodaminen (Absorption of Material by the Living Plant Cell. I. Experiments with Rhodamine). Planta 29, 376.

— 1940. Zur Frage der Stoffaufnahme durch die lebende pflanzliche Zelle. II. Die Aufnahme basischer Farbstoffe und das Permeabilitätsproblem (Absorption of Material by the Living Plant Cell. II. Assimilation of Basic Dyes and Permeability). Flora 134, 159.

Eisenberg, K. R. 1919. Über die Wirkung von Salzen und Ionen auf Bakterien (Effect of Salts and Ions on Bacteria). Zbl. Bakt. I. Orig. 82, 69.

O
Ellinger, H. W. and A. Hirt. 1930. Eine Methode zur Beobachtung lebender Organe mit stärksten Vergrößerungen im Lumineszenzlicht (Intravitalmikroskopie) (A Method for Observation of Living Organs with the Most Powerful Magnification in Luminescence Light - Intravital Microscopy). Abderhalden, Handb. d. biol. Arbeitsmeth. Abt. V, Teil 2/2, 1753.

Elo, J. E. 1937. Vergleichende Permeabilitätsstudien, besonders an niederen Pflanzen (Comparative Permeability Studies, Especially on Lower Plants). Ann. Bot. Soc. Zool.-Bot. Fenn. Vanamo, Tom. 8, 6.

Fischer, A. 1891. Die Plasmolyse der Bakterien (Plasmolysis of Bacteria). Ber. d. Kgl. Sächs. Ges. d. Wiss., math.-physikal. Kl. 43.

— 1895. Untersuchungen "über Bakterien (Studies on Bacteria). Jb. f. wiss. Bot. 27, 1.

— 1900. Die Empfindlichkeit der Bakterienzelle und das baktericide Serum (Sensitivity of the Bacterial Cell and Bactericidal Serum). Z. f. Hyg. 35, 1.

— 1897. Vorlesungen "über Bakterien (Lectures on Bacteria). Gustav Fischer, Jena.

— 1906. "Über Plasmoptyse der Bakterien (Bacterial Plasmoptysis). Ber. der Deutsch. Bot. Ges. 24, 55.

Fischl, V. and E. Schwenk. 1932. Fluoreszenzmikroskopie als chemotherapeutische Forschungsmethode (Fluorescence Microscopy as a Chemotherapeutic Research Method). Klin. Wschr. 11, Teil 1, 1114.

Frey-Wyssling, A. 1938. Submikroskopische Morphologie des Protoplasmas und seiner Derivate (Submicroscopic Morphology of Protoplasm and Its Derivatives). Borntraeger, Berlin.

Gärtner, H. 1939. Wie hoch ist der Gewinn an positiven Tuberkulosepräparaten durch das Fluoreszenzmikroskop? (How High is the Yield of Positive Tuberculous Specimens with the Fluorescence Microscope?). Z. Tuberk. 85, 27.

Gärtner, K. Ein Beitrag zur Färbbarkeit der lebenden und toten Bakterienzelle (Contribution to the Stainability of Living and Dead Bacterial Cells). Z. f. Hyg. 125, 86.

-1943. Die Sulfonamidwirkung im Lichte der Fluoreszenz- und Elektronenmikroskopie (Action of Sulfonamides in the Light of Fluorescence and Electron Microscopy). Zbl. Bakt. I. Orig. 150, 97.

Gassul, R. and Žolkevič, A. 1927. Über Differenzierungsversuche an Bakterienkulturen mit Hilfe der sogen. Woodschen Strahlen (Differentiation Experiments on Bacterial Cultures by Means of the So-called Wood Rays). Zbl. Bakt. I. Orig. 104, 503.

Gehrmann. 1901. "The Effect of Salt Solution and Other Fluids on Bacteria Compared with Serum Reaction." Zbl. Bakt. I. 29, 445.

Gerlach, F. 1937. Zur Virus-Fluoreszenzmikroskopie (Virus Fluorescence Microscopy). Wien. klin. Wschr. 50, 1575.

-1938. "Über Versuche zur Sichtbarmachung und Züchtung spezifischer Mikroorganismen bei Virus-Infektionskrankheiten u. bosartigen Geschwülsten (Experiments on Visualization and Growing of Specific Microorganisms in Virus Infections and Malignant Tumors). Wien. tierärztl. Monatsschr. 25, 165.

Gicklhorn, J. 1914. Über den Einfluss photodynamisch wirksamer Farbstofflösungen auf pflanzliche Zellen und Gewebe (Effect of Photodynamically Active Dye Solutions on Plant Cells and Tissues). Sitzber. Wien. Akad. math.-naturwiss. Kl. 123, I. 1221.

-1927. "Über vitale Kern- und Plasmafärbung an Pflanzenzellen (Vital Nuclear and Plasma Dyeing in Plant Cells). Protoplasma 2, 1.

Gohde, G. 1944. Zur hygienischen Milchbeurteilung mittels Fluoreszenzmikroskopie (Hygienic Evaluation of Milk by Means of Fluorescence Microscopy). Berl. und Münchn. tierärztliche Wschr. Nr. 11/12, 94.

- Goppelsroeder, F. 1901. Capillaranalyse (Capillary Analysis). Basel.
- Graf, F. 1958. Fluoreszenzmikroskopische Untersuchungen zum Nachweis von Bakterien in der Milch (Fluorescence-Microscopic Studies on Detection of Bacteria in Milk). Dissertation, Hannover.
- Gram, Chr. 1884. "Über die isolierte Färbung der Schizomyceten in Schnitt- und Trockenpräparaten (Isolated Staining of Schizomycetes in Section and Dry Preparations). Fortschr. d. Med. 185.
- Hagemann, P. 1937. Fluoreszenzmikroskopische Untersuchungen über Virus und andere Mikroben (Fluorescence-Microscopic Studies on Viruses and Other Microbes). Zbl. Bakt. Parasitenkd. u. Infekt. I. 140, 184.
- 1937. Virus-Fluoreszenzmikroskopie (Virus Fluorescence Microscopy). Münchn. med. Wschr. 20, 761.
- 1937. Fluoreszenzmikroskopischer Nachweis von Leprabakterien im Nasenschleim und im Blut (Fluorescence-Microscopic Detection of Leprosy Bacilli in Nasal Mucus and in Blood). Dtsch. med. Wschr. 63, 514.
- 1938. Fluoreszenzfärbung von Tuberkelbakterien mit Auramin (Fluorescence Dyeing of Tubercle Bacteria with Auramine). München med. Wschr. 85, 1066.
- Haitinger, M. 1930. Ein Fluoreszenzmikroskop mit einfachen Mitteln (A Fluorescence Microscope with Simple Means). Mikrochemie 8, 81.
- 1934. Die Methoden der Fluoreszenzmikroskopie (Methods of Fluorescence Microscopy). Abderhalden, Handb. d. biol. Arbeitsmeth. II, Teil 2, 3307.
- 1938. Fluoreszenzmikroskopie. Ihre Anwendung in der Histologie und Chemie (Fluorescence Microscopy. Its Application in Histology and Chemistry). Leipzig, Akad. Verlagsanstalt.
- 1938. Fluoreszenzmikroskopie (Fluorescence Microscopy). Handb. d. Virusforsch. von Doerr und Hallauer. Springer, Wien.
- Haitinger, M. and R. Schwertner. 1940. Beiträge zur Fluoreszenzmikroskopie in der Bakteriologie (Contributions to Fluorescence Microscopy in Bacteriology). Zbl. Bakt. I. Orig. 145, 141.
- Hamperl, K. 1943. Fluoreszenzmikroskopie. Wesen und Anwendung in der Medizin (Fluorescence Microscopy. Nature and Use in Medicine). Med. Klinik 39, 781.
- Haurowitz, F. 1922. "Über die Differenzierung lebenden und toten Protoplasmas durch Methylgrün. (Differentiation of Living and Dead Protoplasm with Methyl Green). Virchows Arch. f. path. Anat. 242, 345.

- Hefner, H. 1944. Untersuchungen über die Trockenresistenz von Bakterien mit Hilfe der Acridinorangemethode (Studies on Resistance to Drying by Bacteria Using the Acridine Orange Method). Dissertation, Hannover.
- Hegedus, A. 1957. Vitale Färbung von auf farbstoffhaltigen Nährböden gewachsenen Bakterien (Vital Staining of Bacteria Grown on Dye-Containing Nutrient Media). Zbl. f. Bakt. Parasitenkd. und Infekt. I, 158, 99.
- Heimstädt, O. 1911. Das Fluoreszenzmikroskop (The Fluorescence Microscope). Z. wiss. Mikr. 28, 330.
- Hercik, F. 1939. Die fluoreszenzmikroskopische Analyse der α -Strahlenwirkung (Fluorescence-Microscopic Analysis of α -Radiation Effects). Protoplasma 32, 527.
- Herrmann, W. 1936. Der Nachweis von Tuberkelbazillen mit dem Fluoreszenzmikroskop (Identification of Tubercle Bacilli with the Fluorescence Microscope). Dtsch. med. Wschr. 64, 1554.
- Hilbrich, P. 1942. Fluoreszenzmikroskopische Unterscheidung lebender und toter Sporen von Nosema bombycis Nägeli und Nosema apis Zander mit Hilfe der Acridinorangefärbung (Fluorescence-Microscopic Differentiation Between Living and Dead Nosema bombycis Nägeli and Nosema apis Zander Spores by Means of Acridine Orange Dyeing). Dissertation Tierärztl. Hochsch. Hannover.
- Hirt, A. 1939. Intravitalmikroskopie im Lumineszenzlicht (Intravital Microscopy in Luminescence Light). Zeiss-Nachr. 2. Folge, H. 10.
- 1939. Die Lumineszenzmikroskopie und ihre Bedeutung für die medizinische Forschung (Luminescence Microscopy and Its Value in Medical Research). Zeiss-Nachr. 3. Folge, H. 1-5.
- Höfler, K. 1938. Plasmolyse- und Permeabilitätsstudien an Algen und Bakterien (Plasmolysis and Permeability Studies on Algae and Bacteria). Zbl. Bakt. I. 150, 399. (Ref.).
- 1947. Was lehrt die Fluoreszenzmikroskopie von der Plasmapermeabilität und Stoffspeicherung (What Fluorescence Microscopy Teaches About Plasma Permeability and Dye Storage). Mikroskopie 2, 13.
- Jancsó, N. V. 1952. Beobachtung chemotherapeutischer Vorgänge im Fluoreszenzmikroskop (Observation of Chemotherapeutic Processes in the Fluorescence Microscope). Klin. Wschr. 11, 689.
- Jensen, K. A. 1935. Einige neue fluoreszierende Indikatoren (Some New Fluorescing Indicators). Z. analyt. Chemie 94, 177.

Johannes, H. 1939. Beiträge zur Vitalfärbung von Pilzmycelien
(Contributions to the Vital Staining of Fungus Mycelium). Flora,
134, 58.

—1947. Die Vitalfärbung von Phycomyces blakesleeana mit Acridinorange
(Vital Staining of Phycomyces blakesleeana with Acridine Orange).
In press.

Jung, J. W. 1940. Kann die Fluoreszenzmikroskopie den Tierversuch
beim Tuberkelbazillennachweis ersetzen? (Can Fluorescence Microscopy
Replace Animal Experiments for Identifying Tubercle Bacilli?).
Dtsch. Tuberkulosebl. 14, 65.

Jung, H. 1947. Zum Wert der fluoreszenzmikroskopischen Untersuchung
von Milchproben bei Verwendung von Akridinorange als Fluorochrom
(Value of Fluorescence-Microscopic Examination of Milk Samples
with Acridine Orange Used as a Fluorochrome). Dissertation, Hannover.

Kaiserling, C. 1921. Mitteilungen über das Verhalten von Bakterien
im Lumineszenz-Mikroskop. Verhandl. d. Dtsch. Pathol. Ges. 18.
Tagung Jena 12. bis 14. April 1921 (Reports on the Behavior of
Bacteria in the Luminescence Microscope. Proceedings of the German
Pathological Society, 18th Session, Jena, 12 to 14 April 1921), 91,
G. Fischer, Jena.

Keller, Ch. J. 1938. Vereinfachter Nachweis von Tuberkelbazillen im
Fluoreszenzmikroskop (Simplified Detection of Tubercle Bacilli
in Fluorescence Light). Münchn. med. Wschr. 52, 2024.

—1938. Der Nachweis von Diphtheriebazillen mit einem neuen Fluoreszenz-
mikroskop (Identification of Diphtheria Bacilli with a New Fluorescence
Microscope). Wien. med. Wschr. 329.

Köhler, A. 1904. Mikrophotographische Untersuchungen im ultravioletten
Licht (Microphotographic Studies in Ultraviolet Light). Z. wiss.
Mikr. 21, 274.

Kölbel, H. 1947. Untersuchungen über die quantitative Farbstoff-
speicherung von Acridinorange in lebenden und toten Zellen und
ihre Beziehung zu den elektrischen Verhältnissen der lebenden und
toten Plasma-Eiweißkörper (Studies on Quantitative Dye Storage of
Acridine Orange by Living and Dead Cells and Their Connection with
the Electrical Relations of Living and Dead Plasma Proteins).
Dissertation, Hannover.

—1947. Quantitative Untersuchungen über die Farbstoffspeicherung von
Acridinorange in lebenden und toten Hefezellen und ihre Beziehung
zu den elektrischen Verhältnissen der Zelle (Quantitative Studies
on Dye Storage of Acridine Orange in Living and Dead Yeast Cells
and Their Connection with the Electrical Relations of the Cell).
Ztschr. f. Naturforsch. 2 b, 381.

- Küster, E. 1926. "Über vitale Protoplasmafärbung." (Vital Staining of Protoplasm). Z. wiss. Mikr. 43, 376.
- Lasseur, Ph., Lupaix, A., and A. Lecaille. 1931. Fluorescence des corps microbiens. Trav. Labor. Microbiol. Fac. Pharm. Nancy, H. 4, 85.
- Lehmann, H. 1910. "Über ein Filter für ultraviolette Strahlen und seine Anwendung (A Filter for Ultraviolet Rays and Its Application). Physik. Z. 11, 1039.
- 1913. Das Lumineszenzmikroskop, seine Grundlagen und seine Anwendungen (The Luminescence Microscope, Its Principles, and Its Applications). Z. f. Mikr. 30, 417.
- Liesegang, R. 1943. Kreuzkapillaranalyse (Cross Capillary Analysis). Naturwiss. 31, H. 29/30.
- Luyet, B. J. 1937. "The Vitrification of Organic Colloids and of Protoplasm." Biodynamica 29.
- Luyet, B. J. and P. K. Gehenio. 1938. "The Survival of Moss Vitrified in Liquid Air and Its Relation to Water Content." Biodynamica 42.
- May, J. 1947. Einfache Sporenkontrastfärbung mit Oxypyrentrisulfosaure (Simple Spore Contrast Staining with Oxypyrene Trisulfate). Zbl. f. Bakt., Parasitenkd., Infekt. u. Hyg. 152, 56.
- Metzner, P. 1919. "Über die Wirkung photodynamischer Stoffe auf Spirillum volutans und die Beziehungen der photodynamischen Erscheinung zur Phototaxis (Effect of Photodynamic Substances on Spirillum volutans and the Relationship Between Photodynamic Phenomena and Phototaxis). I. Bioch. Z. 101, 33.
- 1924. Zur Kenntnis der photodynamischen Erscheinung. III. Über die Bindung der wirksamen Farbstoffe in der Zelle (Photodynamic Phenomena. III. Binding of Active Dye in the Cell). Biochem. Z. 148, 490.
- 1928. Ein einfaches Fluoreszenzmikroskop (A Simple Fluorescence Microscope). Z. wiss. Mikr. 45, 51.
- 1930. Einfache Einrichtungen zur Fluoreszenzmikroskopie und Fluoreszenzphotographie (Simple Devices for Fluorescence Microscopy and Fluorescence Photography). Biol. gener. 6, 415 and Mikrochemie 9, 72 (1931).
- Meyer zu Schweicheln. 1943. "Über Vitalfärbung von Gonokokken (Vital Staining of Gonococci). Arch. f. Dermat. u. Syph. 163, 464.

Naylor, L. L. 1926. "The Hydrogen-Ion Concentration and the Staining of Sections of Plant Tissue." Amer. Jour. of Bot. 13, 265.

Nordmeyer, K. 1947. Eine neue Methode zur Bestimmung des isoelektrischen Punktes von Bakterien (A New Method of Determining the Isoelectric Point of Bacteria). Zbl. f. Bakt., Parasitenkd., Infekt. u. Hyg. 152, 54.

Pesch, K. L. 1939. Fluoreszenz-mikroskopischer Nachweis von Bakteriensporen (Demonstration in der Sitzung vom 12. Dec. 1938 der Berliner Mikrobiologischen Gesellschaft) (Fluorescence-Microscopic Detection of Bacterial Spores [Demonstration in the 12 December 1938 Meeting of the Berlin Microbiological Society]). Referat Berl. Tierärztl. Wschr. Nr. 7, 114.

Pfeffer, W. 1886. Untersuchungen aus dem Botanischen Institut zu Tübingen (Research Conducted by the Botanical Institute of Tübingen). Bd. II. Verlag Engelmann, Leipzig.

Pickarski, J. and H. Ruska. 1939. "Übermikroskopische Untersuchungen an Bakterien unter besonderer Berücksichtigung der sogenannten Nucleoide (Supermicroscopic Studies on Bacteria with Particular Reference to the So-called Nucleoids). Arch. f. Mikrobiol. 10, 302.

Pischinger, A. 1926. Die Lage des IEP histologischer Elemente als Ursache ihrer verschiedenen Färbbarkeit (The Position of the IEP of Histologic Elements as a Cause of Their Variable Stainability). Z. f. Zellforsch. u. mikr. Anatomie 3, 169.

— 1927. "Diffusibilität und Dispersität von Farbstoffen zur Färbung bei verschiedenen H-Ionenkonzentrationen (Diffusibility and Dispersion of Dyes with Different H-Ion Concentrations). Arch. f. Zellforsch. u. mikr. Anatomie 5, 347.

Pringsheim, P. 1926. Fluoreszenz und Phosphoreszenz (Fluorescence and Phosphorescence). Springer, Berlin.

Provazek, S. V. 1914. "Über Fluoreszenz der Zellen (Cell Fluorescence). Kleine Welt 6.

Raichel, B. 1926. "Über den Einfluss osmotisch wirksamer Mittel auf die Bakterienzelle (Effect of Osmotically Active Agents on the Bacterial Cell). Arch. f. Protistenkd. 63, 333.

Raffelt, G. 1944. Untersuchungen über den Einfluss des Milieuwechsels auf die Bakterienzelle (Studies on the Effect of Change in Medium on the Bacterial Cell). Dissertation, Hannover.

Reichert, C. 1911. Das Fluoreszenzmikroskop (The Fluorescence-Microscope). Physik. Z. 12, 1010.

- 1919. Sichtbarmachung der Geisseln und Geisselbewegungen der Bakterien (Visualization of Flagella and Movement of Flagella in Bacteria). Zbl. Bakt. I. 51, (Orig.), 14.
- Rühl, H. 1940. Erfahrungen über den Tuberkelbazillennachweis mit dem Fluoreszenzmikroskop (Fluorescence-Microscopic Detection of Tubercle Bacilli). Wien. klin. Wschr. 522 and Wien. Arch. inn. Med. 55, 344.
- Schöcklin, E. 1933. Über die Nuklealreaktion bei Hefen (Nuclear Reaction in Yeast). Zbl. Bakt. II. Abt. 86, 304.
- Schouschal, Ch. and S. Strugger. 1943. Eine neue Methode zur Vitalbeobachtung der Mikroorganismen im Erdboden (A New Method of Vital Observation of Microorganisms in the Soil). Naturwiss. 31, H. 25/26.
- Ružička, V. 1904. Über tinctorielle Differenzen zwischen lebendem und abgestorbenem Protoplasma (Tinctorial Differences Between Living and Dead Protoplasm). Arch. ges. Physiol. 107, 497.
- Savini. 1909. Beitrag zur experimentellen Biologie des z-Bacillus und seine Beziehungen zum Keuchhusten (Contribution to the Experimental Biology of the z-Bacillus and Its Connection with Whooping Cough). Zbl. Bakt. I. 50 (Orig.), 562.
- Schallock, G. 1940. Vereinfachter Nachweis von Tuberkelbazillen in histologischen Schnitten durch die Fluoreszenzmethode nach Hagemann (Simplified Identification of Tubercle Bacilli in Histological Sections by Hagemann's Fluorescence Method). Münchn. med. Wschr. 87, 102.
- Scheibe, G. 1938. Reversible Polymerisation als Ursache neuartiger Absorptionsbanden von Farbstoffen (Reversible Polymerization as a Cause of New Absorption Bands of Dyes). Kolloid-Ztschr. 82, 1.
- 1939. Die Stereoisomerie organischer Farbstoffe und ihr Zusammenhang mit Konstitution und Eigenschaften reversibel polymerer Farbstoffe (Stereoisomerism of Organic Dyes and Their Relation to the Constitution and Characteristics of Reversible Polymeric Dyes). Angew. Chemie 52, 631.
- Schneider, G. H. 1944. Untersuchungen über die bakterizide Wirksamkeit von Methyl- und Äthylalkohol auf die Bakterien mit Hilfe der Acridinorange-Methodik (Studies on the Bactericidal Effects of Methyl and Ethyl Alcohol on Bacteria Using the Acridine Orange Method). Dissertation, Tierärztliche Hochschule Hannover.
- Schönberg, F. 1946. Zur Brauchbarkeit der fluoreszenzmikroskopischen Untersuchung des vitalgefärbten Milchsedimentes (Usefulness of Fluorescence-Microscopic Examination of Vitally Stained Milk Sediment). Dtsch. Tierärztl. Wschr. 53, 147.

Schumacher, J. 1923. Würzburger Mikrobiologentagung Juni 1922, Vortrag Schumacher (Würzburg Congress of Microbiologists, June 1922, Schumacher's Proposal). Zbl. Bakt. Abt. I. Orig. 89, 206.

-1925. Worauf beruht die spirillocide Wirkung des Salvarsans? (What is the Basis of the Spirillocide Effect of Salvarsan?). Arch. f. Dermat. u. Syph. 149, 13.

Schumacher, W., 1933. Untersuchungen über die Wanderung des Fluoresceins in den Siebröhren (Studies on the Migration of Fluorescein in Sieve Tubes). Jb. f. wiss. Bot. 77, 665.

-1936. Untersuchungen über die Wanderung des Fluoresceins in den Haaren von Cucurbita Pepo (Studies on the Migration of Fluorescein in Cucurbita pepo Hairs). Jb. f. wiss. Bot. 82, 507.

Stickl, O. and K. Gartner. 1943. Die Wirkungsweise der Sulfonamide und ihre chemotherapeutische Anwendung bei Ruhr (The Mechanism of Action of the Sulfonamides and Their Chemotherapeutic Use in Dysentery). Z. f. Hyg. 125, 226.

Strugger, S. 1931. Zur Analyse der Vitalfärbung pflanzlicher Zellen mit Erythrosin (Analysis of Vital Staining of Plant Cells with Erythrosin). Ber. d. Dtsch. Bot. Ges. 49, 453.

-1935. Praktikum der Zell- und Gewebephysiologie der Pflanze (Manual of Cell and Tissue Physiology of Plants). Borntraeger, Berlin. 2nd ed. in press. Springer, Heidelberg (1948).

-1938. Die Vitalfärbung des Protoplasmas mit Rhodamin B und 6 G (Vital Staining of Protoplasm with Rhodamine B and 6G). Protoplasma 30, 85.

-1938. Fluoreszenzmikroskopische Untersuchungen über die Speicherung und Wanderung des Fluoreszeinkaliums in pflanzlichen Geweben (Fluorescence-Microscopic Studies on the Storage and Migration of Potassium Fluorescein in Plant Tissues). Flora 132, 253.

-1939. Die lumineszenzmikroskopische Analyse des Transpirationsstromes in Parenchymen. III. Untersuchungen an Helxine Soleirolii Req. (Luminescence-Microscopic Analysis of Transpiration Flow in Helxine soleirolii Req.). Biol. Zbl. 59, 409.

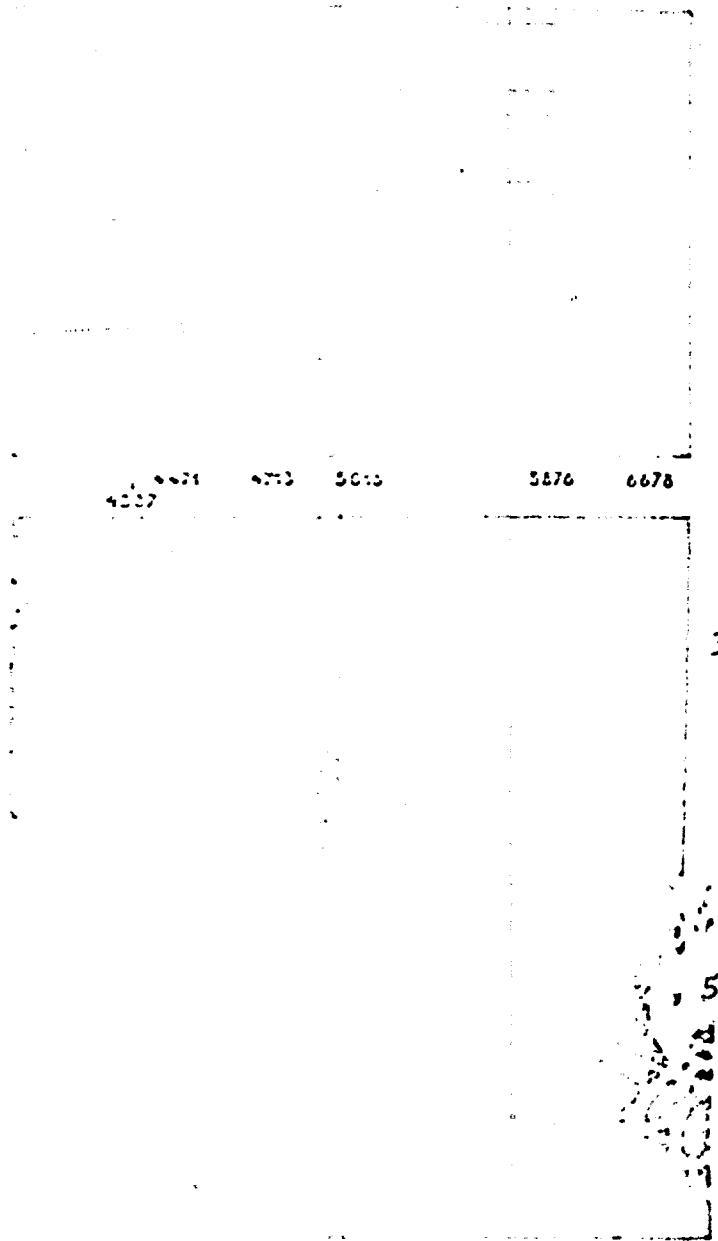
-1940. Fluoreszenzmikroskopische Untersuchungen über die Aufnahme und Speicherung des Acridinorange durch lebende und tote Pflanzenzellen (Fluorescence-Microscopic Studies on the Absorption and Storage of Acridine Orange by Living and Dead Plant Cells). Jenaische Ztschr. f. Naturwiss. 73, 97.

- 1940. Die Vitalfärbung der Chromosomen. Dtsch. tierärztl. Wschr. 48, 645.
- 1940. Die Kultur von Didymium nigripes aus Myxamöben mit vital gefärbtem Plasma und Zellkernen (Cultivation of Didymium nigripes from Myxamoebas with Vitality Stained Plasma and Nuclei). Z. wiss. Mikr. u. mikr. Techn. 57, 415.
- 1941. Die fluoreszenzmikroskopische Unterscheidung lebender und toter Zellen mit Hilfe der Acridinorange-Färbung (Fluorescence-Microscopic Differentiation Between Living and Dead Cells Using Acridine Orange Staining). Dtsch. tierärztl. Wschr. 49, 525.
- 1941. Zellphysiologische Studien mit Fluoreszenzindikatoren. I. Basische, zweifarbig Indikatoren (Cell Physiological Studies with Fluorescence Indicators. I. Basic, Two-Color Indicators). Flora 135, 101.
- 1942. Ein neues Verfahren zur Färbung von Bakterien (A New Dyeing Technique for Bacteria). Berl. und Münchn. tierärztl. Wschr. 42, 253.
- 1942. Neues über die Fluoreszenzfärbung toter und lebender Bakterien (New Developments on Fluorescence Staining of Dead and Living Bacteria). Dtsch. tierärztl. Wschr. 50, 51.
- 1942. Fluoreszenzmikroskopische Beobachtungen über das Eindringen des Prontosil solubile in lebende Bakterien- und Hefezellen (Fluorescence-Microscopic Observations on the Penetration of Soluble Prontosil into Bacterial and Yeast Cells). Ibid., 321.
- 1943. Untersuchungen über die vitale Fluorochromierung der Hefezelle (Studies on Vital Fluorochroming of Yeast Cells). Flora 137, 73.
- 1944. Zum heutigen Stand der Acridinorange-Färbung lebender und toter Bakterien auch für die Milchuntersuchung (Present Status of Acridine Orange Staining of Living and Dead Bacteria for Milk Research). Z. f. Fleisch- und Milchhyg. 54, 161.
- 1946. Die Fluorochromierung von Bakteriensporen mit Brillantsulfoflavin (Fluorochroming of Bacterial Spores with Brilliant Sulfoflavin) Dtsch. tierärztl. Wschr. 53, 145.
- 1947. Der fluoreszenzmikroskopische Nachweis von Trypanosomen im Blute (Fluorescence-Microscopic Detection of Trypanosomes in Blood). Ibid. 54, 161.
- 1948. "Fluorescence Microscope Examination of Bacteria in Soil." Canadian Jour. of Research 26, 186.

- 1947. Zellphysiologie und Protoplasmatik (Pflanzenzellen) (Cell Physiology and Protoplasm Research [Plant Cells]). Naturforsch. u. Medizin in Deutschld. 1959-1946, FIAT-Review of German Science.
- Strugger, S. and P. Hilbrich. 1942. Die fluoreszenzmikroskopische Unterscheidung lebender und toter Bakterienzellen mit Hilfe der Acridinorange-Färbung (Fluorescence-Microscopic Differentiation Between Living and Dead Bacterial Cells with Acridine Orange Staining). Dtsch. tierärztl. Wschr. 50, 121.
- Strugger, S. and G. Rosenberger. 1944. Vitalfärbung der Ziegenspermatozoen mit Acridinorange (Vital Staining of Goat Spermatozoa with Acridine Orange). Dtsch. tierärztl. Wschr. and Dtsch. tierärztl. Rundschau, Nr. 39/40, 52/50. Janrg. 357.
- Stübel, H. Die Fluoreszenz tierischer Gewebe in ultraviolettem Licht (Fluorescence of Animal Tissue in Ultraviolet Light). Pflügers Arch. Physiol. 142, 1.
- Tappeiner, Fr. H. and A. Jodlbauer. 1904. Über die Wirkung der photodynamischen (fluoreszierenden) Stoffe auf Protozoen und Enzyme (Effects of Photodynamic (Fluorescing) Substances on Protozoans and Enzymes). Dtsch. Arch. f. klin. Med. 80, 427.
- Voigt, K. 1927. Über das Verhalten der Hefe zur Nuklealfärbung (Reaction of Yeast to Nuclear Dyeing). Ges. exp. Medizin 55, 569.
- Vonkennel and Wiedemann. 1944. Fluoreszenzmikroskopische Untersuchungen an Gonokokken nach Sulfonamideinwirkung (Fluorescence-Microscopic Studies on Gonococci After Treatment with Sulfonamides). Dtsch. med. Wschr. 70, 529.
- Wasitzki, R. 1913. Das Fluoreszenzmikroskop in der Pharmakognosie (The Fluorescence Microscope in Pharmacognosy). Pharmaz. Post, Heft 82.
- Weigert. 1875. Jahresbericht der Schles. Ges. f. vaterland. Kultur am 10. 12. 1875 (Annual Report of the Schleswig Society for Native Culture, 10 December 1875), 229.
- Willschke, A. 1914. Über die Fluoreszenz der Chlorophyllkomponenten (Fluorescence of Chlorophyll Components). Z. wiss. Mikr. 31, 338.
- Winogradsky, S. 1925. Etudes sur la microbiologie du sol. I. Ann. Inst. Pasteur 39, 229.
- 1928. Begriff der "autochthonen" Mikroorganismenflora (Concept of "Autochthonous" Microflora). J. r. Acad. Sci. Paris 187, 161.
- 1929. Etudes sur la microbiologie du sol. II. Ann. Inst. Pasteur 40, 455.

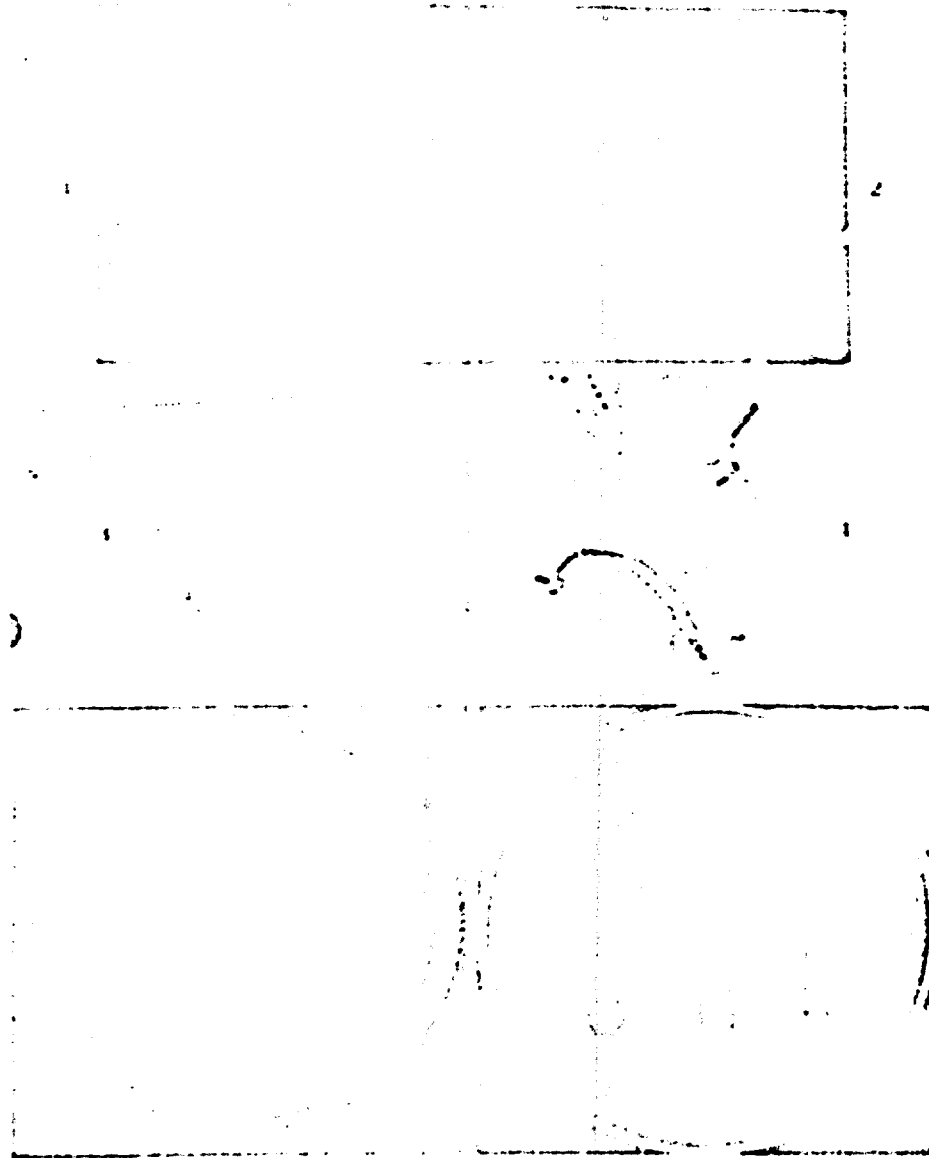
Wohlfell, H. 1927. Versuche über die Wirkung vorwiegend osmotischer Prozesse auf Typhus-Coli-Bacillengemische mit besonderer Berücksichtigung der Agglutination (Experiments on the Effect of Predominantly Osmotic Processes on Mixtures of Typhus and Coli Bacilli with Special Reference to Agglutination). Zbl. Bakt. I. 105, (orig.), 15.

Plate I*



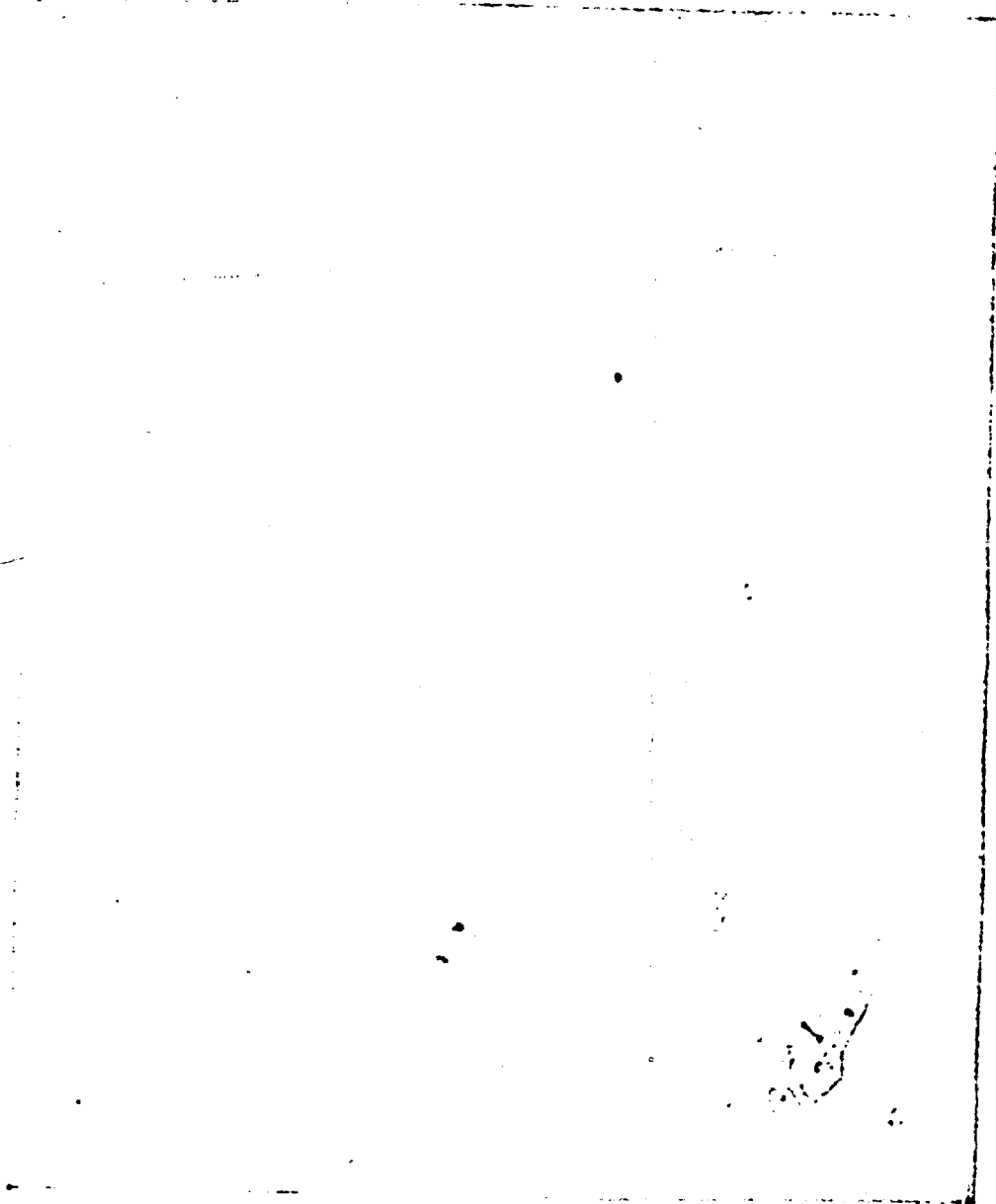
*Explanations on p. 167.

Plate II



C

Plate III



C

Plate I

- 1 - Fluorescence spectra of several substances, from top: (1) aqueous solution of 1:1000 5-oxy-5,8,10-pyrene trisulfate (sodium oxy-pyrene trisulfate) (time of exposure 1 minute); (2) aqueous solution of 1:10,000 acridine orange (tetramethyldiamino acridine) (time of exposure 30 minutes); (3) aqueous solution of 1:100 acridine orange (time of exposure 30 minutes); (4) 1:1000 auramine adsorbed on filter paper (time of exposure 5 minutes); (5) helium light as wave normal

These fluorescence spectra were excited with blue light $< 4800 \text{ \AA}$. Taken by the Physical Institute, Hannover Technical College, Prof. Bartels, director.

- 2 - Heat-fixed smear preparation of Staphylococcus aureus fluorochromed with 1:1000 auramine, duration of dyeing 1 minute
- 3 - Heat-fixed smear preparation of Bacterium pullorum fluorochromed with 1:1000 rivanol, duration of dyeing 1 minute
- 4 - Heat-fixed smear of sordes on a tooth fluorochromed with 1:1000 rivanol, duration of dyeing 5 minutes
- 5 - Smear of Bacillus mycoides, heat-fixed. The spores were electively fluorochromed with brilliant sulfoflavin FF.

Plate II

- 1 - Mycobacterium tuberculosis var. avium fluorochromed with auramine (Hagemann's method)
- 2 - Yeast cell suspension fluorochromed with 1:5000 acridine orange in tap water. Both brightly shining yeast cells have shiny copper-red fluorescing protoplasm and are dead. The other yeast cells have green fluorescing protoplasm and are living. The vacuoles appear dark. Individual crumb-like inclusions fluoresce copper red (Strugger, 1942).
- 3 - Bright-field photograph of a microculture (gum arabic) of Bacillus bombycis cells vitally and lethally stained with acridine orange. The copper-red fluorescing bacilli are darker than the green fluorescing ones. Three living and two dead cells (initial picture at 11:30 hours).
- 4 - The same place photographed at 16:30 hours. Beautiful chains of green fluorescing cells have formed, partly projecting into the space. The copper-red fluorescing bacilli are completely unchanged (Strugger and Milbrich, 1942).

- C
- 5 - Bacillus bombycis culture on meat agar with 1:5000 acridine orange added. Strong inhibition.
 - 6 - Bacillus bombycis culture on meat agar with 1:1000 acridine orange added. Inhibition clearly visible (Strugger and Hilbrich, 1942).

Table III

- 1-4 - Soil particles from a good garden soil fluorochromed with acridine orange according to instructions. The humus substances have adsorbed the acridine orange cations electrostatically with such intensity that the soil particles fluoresce copper red. The autochthonous soil bacteria resting on the humus layers were vitally fluorochromed with acridine orange and fluoresce green. Fig. 4 shows a rather large zooglea formed from cocci (drawn from nature by Ch. Rouschal).

INDEX*

- Absorption filter for blue-light fluorescence microscopy, 8-12, 15
Absorption filter for UV-light microscopy, 14*, 15
Acid fuchsin, 24*
Acridine, 19, 48, 59
Acridine orange, 5, 19, 20, 24, 25*, 30, 31, 33, 38, 44 ff, 48*ff
Acridine orange, affinity for protoplasmatic proteins, 45ff, 66;
analysis of concentration effect in living and dead cells, 61,
64-66, 74*, 75*, 76*, 77; basic nature, 48; cataphoresis, 51;
characteristics as a fluorochrome, 50ff; chemical characteris-
tics of, 48*; concentration and fluorescence color, 50, 51;
concentration effect, 46, 50, 51, 52, 54, 55, 63ff; differen-
tiation between living and dead bacterial spores, 114; dif-
ferentiation between living and dead protoplasts, 57-60, 61ff,
66ff, 83ff; dissociation curve, 52*, 54, 60; fluorescence in-
dicator, 50, 51; fluorescence spectrum, 50, 51; fluorochroming,
duration of, 75*; fluorochroming of Azotobacter, 115, 116*, 117;
fluorochroming of Bacillus species, 113, 114; fluorochroming
of bacterial suspensions, 83ff, 133; fluorochroming of Bacterium
coli, 102, 103, 104*; fluorochroming of blood, 86, 87; fluoro-
chroming of gonococci, 105-107; fluorochroming of higher plant
cells, 57ff; fluorochroming of milk centrifugates, 87; fluoro-
chroming of molds, 25*, 54, 55*, 56; fluorochroming of mucors,
47, 81, 82*, 83*; fluorochroming of Mycobacterium tuberculosis,
117, 118*, 119, 128*, 129; fluorochroming of Nosema spores, 119;
fluorochroming of pus, 88, 105; fluorochroming of Sarcina, 113;
fluorochroming of spermatozoa, 46, 101; fluorochroming of spu-
tum, 88; fluorochroming of Staphylococcus, 107-112; fluoro-
chroming of Streptococcus, 113; fluorochroming of trypanosomes,
87; fluorochroming of yeast cells, 74*-76*, 77, 79, 80, 81*ff;
fluorochroming, rapid procedure for, 85-88; general aspects of
fluorochroming living cells, 45-47; intrinsic color and pH
value of, 49; intrinsic color of, 49; lipid solubility, 54, 56;
literature on vital staining, 46, 47; method of fluorochroming
soil bacteria, 132ff; pH dependence of vital fluorochroming,
59-61, 76, 77; pH value and fluorescence, 50, 51, 52*, 54;
storage in cellulose, 54, 55*, 56; storage in gelatin, 25*, 26;
synthesis, 48; systematic evaluation of fluorochroming, 66, 68ff,
96ff; theory of fluorochroming, 67-71; use for titration, 51
Acridine orange method, 44ff
Acridine red, 19, 33, 69
Acridine yellow, 33, 41, 69, 88
Acridine, 48, 69, 105

* Pages marked with an asterisk contain figures with indexed item.

Aesculin, 22
 Alcohol effect on tubercle bacteria, 128*
Allium cepa, 57ff, 129
 Analysis of vitality of the bacterial cell, 83ff
 Animal experiments with vitally fluorochromed bacteria, 93, 126
 Anthracene preparation for adjustment of a fluorescence microscope, 7, 9
 Apparent death, 71
 Applicability of acridine orange method to various bacterial forms, 101ff
 Arc lamp, 7, 8, 10, 13*, 14*
 Artificial inoculation with vitally stained sperm, 66, 101
 Asphyxia and vital fluorochroming, 108-110
 Atebrin, 48, 69
 Auramine, 20, 22, 23*, 28, 33, 36, 39-43, 88
 Aurazine C, 35
 Autochthonous soil bacteria, 132, 135-150
Azotobacter, 27, 115-117, 123*

Bacillus bombrois, 30, 37, 94, 95, 99, 113
Bacillus mesentericus, 27, 28, 38, 113
Bacillus mycoloides, 37, 113
Bacillus subtilis, 27, 99, 113
 Bacterial content of soils, 141-148
 Bacterial forms in soil, 136-141, 146, 147
 Bacterial pure cultures, checking of, 86
 Bacterial spores, fluorochroming with acridine orange, 86, 114, 136; fluorochroming with brilliant sulfoflavin, 36-38, 140, 141
 Bacterial spores in soil, 138, 140
Bacterium avium, 105, 126
Bacterium coli, 50, 68, 98, 102-105, 121, 122*, 124-129
Bacterium enteritidis, 88, 96, 99
Bacterium fluorescens, 105, 124*, 128
Bacterium paratyphi, 105
Bacterium proteus, 105, 124
Bacterium pyocyaneum, 30, 100, 105
Bacterium rhusiopathiae suis, 94
 Beer's law, 74
 Benzoflavin, 19, 33, 69
 Berberine sulfate, 28, 33, 38
 Blepharoplast, 87
 Blood, 43, 65-87
 Blood smears, 43
 Blue-light fluorescence microscope, use of, 15
 Brilliant sulfoflavin, 5, 36-38, 140
 Buffer solutions, preparation of, 27

Calcium chloride effect on bacteria, 124
 Canary virus, 44
 Capillary analysis, 17, 20, 48
 Capsules, 86, 89, 116
 Carbofuchsin, 36, 38
 Catalase, 110
 Cataphoresis, 51, 68
 Causative agent of udder inflammation, 88
 Cell membrane, discharge point, 59; electric charge of, 59, 60, 68, 69; fluorochroming of, 57-60, 61, 63, 68, 73, 80, 81, 82*, 83*, 86, 103, 107ff
 Cell physiology of bacteria, 119ff
 Cellulose, electric charge of, 23, 54, 56
 Cellulose fibers, 22, 23*, 54, 55*, 56
 Chemoluminescence, 3
 Chlorophyll, fluorescence in microorganisms (soil), 139
 Chromatin, 59
 Chromomeres, 62
 Chromonema, 62
 Chromosomes, 46, 66
 Chromosome equivalents (yeast), 73
 Chromotropic acid, 22
 Coagulation structure of protoplasm, 59, 103
 Colorimetry, 23-25*, 54, 55*, 74, 75*, 76*, 77
 Congo red, 88
 Copulation of myxamoebas, 46
 Copulation of Phycomyces mycelium, 83
 Corioflavin, 33
 Coriphosphine, 33, 36, 37, 140
 Coumarin, 22
 Coumaric acid, 22
 Counting eyepiece, Ehrlich's, 80, 142
 Counting of living and dead yeast cells, 80, 81*
 Counting of soil bacteria, 141-146
 Counting of vitally fluorochromed bacteria in living and dead cells, 98-98, 104*, 105, 115, 118*, 124*, 125*, 127*, 128*, 129, 141-145
 Counting procedure, Conn and Winogradsky's, 132, 141
 Cultivation and animal experiments and interpretation of vitality, 84, 85
 Cultivation of vitally fluorochromed microorganisms, 46, 77, 79, 81, 82*, 83, 92-95, 108, 118, 150
 Cultivation of vitally fluorochromed yeast cells, 77, 79
 Cultivation of vitally stained mucors, 81, 82*, 83*
 Cytoplasm, vital fluorochroming of, 57-59, 64-66, 73, 80, 82, 83*, 87, 88

Degeneration of bacterial protoplasts, 129, 130*
 Diachrome, 17, 24, 29, 44, 47
 Diachroming, 17, 24*, 29, 44
 3,6-Diaminoacridine, 33
Didymium nigripes, 46
 Differentiation between living and dead bacterial cells, 89ff
 Differentiation between living and dead mucor spores, 82, 83*
 Differentiation between living and dead spirilla, 99, 100
 Differentiation between living and dead yeast cells, 72, 73
 Differentiation experiment in gelatin, 21, 22
 Differentiation of acid-resistant bacteria with HCl-alcohol, 39, 41-43
 Differentiation of acid-resistant bacteria with hot water, 38
 Dimethyl-m-xylylene-diamine, 48
 1,3-dioxynaphthalene-5,7-disulfate, 35
 Disinfection agent, effect, and fluorochroming of, 101, 119
 Dissociation and pH value, 17, 18*, 19, 21*, 52
 Distribution of soil bacteria, 136-139, 141
 Drying media, 128, 129
 Dye storage, pH dependence of, 23-25*, 27ff

 Ectromelia virus, 44
 Effect of acridine orange on yeast cells, 77, 79, 90, 81*
 Effect of dryness on the bacterial content of soil, 145, 147, 148
 Electroadsorption, 23, 24, 25*, 26, 29, 31, 54-56, 60, 67, 68
 Electron microscopy, 101
 Eosin, 22, 35, 44
 Epithelial cells, 106
 Epithelioid cells, 88
 Erythrosin, 22, 44, 132
 Erythrosin staining of soil bacteria after Conn, 132
 Estimation of soil bacterial content, 149
 Estimation of soil humus content, 147, 148
 Euchrysin, 33
 Evaluation of vitality of yeast cells, 73, 74
 Exciting light filter for blue-light fluorescence microscopy, 7-9, 11, 12, 14*
 Extinction measurements, 23-25*, 54, 55, 74-76*, 77
 Extraction of fluorochrome with benzene or toluene, 19, 20, 53, 54

 Fixation of bacterial smears, 26, 30, 31, 33, 37
 Flagellum of trypanosomes, 87
 Flavophosphine, 36
 Fluorescein, 22, 69; potassium, 30, 35, 45; sodium, 5
 Fluorescence analysis, sensitivity of, 4, 76*, 77
 Fluorescence, chlorophyll, 139; primary, 16; principles of, 3, 4; secondary, 16; theory of, 4

Fluorescence color and adsorption, 20
 Fluorescence color and concentration, 20, 50, 51
 Fluorescence color and degree of dispersion, 21, 22
 Fluorescence color in different solvents, 20
 Fluorescence color, pH dependence of, 18*, 19, 21*
 Fluorescence excitation and wavelength of light, 3
 Fluorescence indicators, anodic, 21*; cathodic, 18, 19, 25, 26, 51, 52, 54; one-color, 18, 19, 21, 52; two-color, 18, 21, 25
 Fluorescence microscope, blue-light, 8ff; construction of, 6*, 13*, 14*; historical, 2ff; light sources of, 6-15; objective and eyepieces, 7; preparation of a test specimen, 7; principle of, 3-14; UV light, 13*, 14
 Fluorescence-microscopic analysis of soil, 131ff
 Fluorescence-microscopic analysis of soil for algae, 139
 Fluorescence-microscopic counting of soil bacteria, 141-145
 Fluorescence quenching, 29, 82, 83, 109-114
 Fluorescence-quenching pigment in Phycomyces spores, 82, 83
 Fluorochrome, 16ff, 45, 47ff; anodic, 17, 20, 21*, 24, 27ff, 35; cathodic, 17, 18*, 19, 20, 24, 27ff, 33, 45; electroneutral, 17, 21, 27; lipophilic, 17, 19, 20, 21, 25, 26, 32
 Fluorochroming, 16ff, 42ff; acid-resistant bacteria, 38ff, 117-119; bacterial smears, theory of, 22ff, 31ff; bacterial spores, 36-38, 137, 138; blood smears, 43; fixed bacterial smears, 26ff; living microorganisms, 44ff; membrane substance, 22, 23*; Micrococcus gonorrhoeae, 34, 105, 106*, 107; pH dependence of, 26ff, 60, 62, 77; proteins, 24*, 25*, 26, 57; soil suspensions with acridine orange, 132ff; trypanosomes, 43, 87; tubercle bacteria, 38ff, 117-119; tubercle bacteria by Hagemann's method, 38, 39; tubercle bacteria by Herrmann's method, 40; tubercle bacteria in tissue sections by Bachmann and Finke's method, 42; tubercle bacteria in tissue sections by Haitinger and Schwertner's method, 41, 42; vertebrates, 47; virus bodies, 44
 Fluoroscopy of soil, 131ff
 Freezing of protoplasm, 70
 Frog, 47
 Fruiting bodies of myxomycetes, 46
 Fungus spores in soil, 139

 Galt streptococci, 88
 Gelatin as a standard substance, 24*, 25, 26, 56
 Gelatin-benzene model, 25, 26
 Gelatin, isoelectric point of, 24*, 25, 26, 57
 Gelatin-toluene model, 56, 57
 Germination of vitally fluorochromed Phycomyces spores, 82*, 83*
 Glycerin effect on bacteria, 122, 124
 Gonococci, 34, 35, 88, 105, 106*, 107

Gram-negative bacteria, 92, 101, 102, 121
 Gram-positive bacteria, 90, 101, 107ff, 121
 Grape sugar effect on bacteria, 121, 122*, 124*
 Growth of vitally fluorochromed bacteria, 92-95, 116, 118
 Growth of vitally fluorochromed yeast cells, 77, 79
 Gum arabic nutrient medium for microcultures, 93

 Humus, adsorption of acridine orange cations on, 132, 133, 147

 Identification of the bacterial nature of structures in soil
 fluorochromable with acridine orange, 135-137
 Immersion oil for fluorescence microscopy, 7
 Inability to grow and death of the bacterial cell, 84
 Inclusion body effect on vitally fluorochromed bacteria, 91
 Injury due to hypertonicity, 96, 122, 124, 130
 Injury due to hypotonicity, 92
 Instructions for fluorochroming of smears with anodic fluorochromes,
 35
 Instructions for fluorochroming of smears with cathodic fluorochromes,
 33, 34
 Instructions for fluorochroming of yeast cells, 72ff
 Intraplasmatic dye concentration in vital staining, 42, 45, 47, 64,
 67, 74*-76*, 77
 Intravital microscopy in luminescence light, 45ff
 Isoelectric point, 24*, 25*, 26, 29-31, 57, 60, 67-69, 77
 Isoelectric point, determination of, 24*, 25*, 26, 30, 31, 66, 67,
 68
 Isoelectric point of bacterial protoplasts, 28, 31

 Karyolymph, 59, 62
 Karyosome, 87
 Karyotin, 59, 62

 Langhan's giant cells, 88
 Latent life, 70
 Leprosy bacilli, 38, 39
 Leukocyte nuclei, 106
 Lichen, 70
 Light green, 5
 Lipid solubility of fluorochromes, 19-21, 25, 26, 54, 56
 Liquid paraffin as a medium for fluorescence-microscopic examination
 of bacteria, 33, 34, 39, 102, 134
 Löffler's methylene blue, 40
 Low-voltage lamp, 8, 10
 Lymph, 85
 Lymphocytes, 88

Macrocultivation of vitally fluorochromed bacteria, 95
 Mass of bacteria in soil, 146
 Medium effects on bacteria, 92, 94, 95, 98, 104*, 105, 114, 124*, 125*
 Membrane effect of vitally fluorochromed bacteria, 90, 91, 107ff
 Membrane of bacterial cell, 89-91, 103, 107ff
 Mercury-vapor high-pressure lamp, 8, 10, 13
 Mercury-vapor lamp, 8, 10, 13, 15
 Metachromatic substances (yeast), 73
 Method of counting soil bacteria in fluorochromed state, 141-144
 Method of fluorescence-microscopic examination of fluorochromed soil samples, 133-135
 Method of fluorochroming of bacterial spores in soil, 140
 Method of soil fluorochroming, 133-135
 Method, Winogradsky's, for observing soil bacteria, 132
 Methylene blue, 5, 36, 40, 84
 Methylene blue-neutral red method, Rozicka's, 84
 Methyl green, 84
 Methyl green method, Haurowitz', 84
 Methyl orange, 5
 β -methylumbelliferon, 22
Micrococcus aureus, 107
Micrococcus gonorrhoeae, 34, 88, 105-107
 Microcultivation of vitally fluorochromed bacteria, 93, 94, 113, 138, 150; of yeast cells, 77, 79
 Microorganisms in soil, 131ff
 Microscopic analysis of soil bacteria, 131ff
 Milk, 85, 87, 88
 Milk centrifugate, 87
 Milk sediment, cell analysis of, 88
 Model experiments in fluorochroming fixed bacterial smears, 22ff
 Moss, 70
 Mouse, 47
 Mousepox virus, 44
 Mucor, 81, 82*, 83*
 Mucous membranes, 86, 115
 Myxamoebas, 46
Mycobacterium tuberculosis, 38ff, 88, 117, 118*, 119, 126, 128*, 129
 Neosalvarsan, 84
 Neosalvarsan-silver method, Schumacher's, 84
 Neutral red, 5, 19, 83
Nosema apis, 119
Nosema bombycis, 119
 Nuclear framework, 59-62
 Nuclear sap, 59

Nucleoids, 102, 114
 Nucleolus, 62
 Nucleus, Phycomyces blakesleeianus, 81, 82*, 83*
 Nucleus, vital fluorochroming of, 57-60, 64-66, 73, 79, 81, 82*, 83*, 87, 88
 Nucleus, yeast cell, 73

 Oligodynamic effects, 80, 98, 105, 124, 130
 Osmic acid, 84

 Permeability, 121, 123
 Phagocytosis, 68
 Phloxin, 22
 Phosphine 2B, 33
 Phosphine N, 33
 Phosphorescence, 4
 Photoluminescence, 3
 Photometry, 24*, 25*, 54, 55*, 74*-76*, 77
Phycomyces blakesleeianus, 47, 81ff
 Plasmodium, 46
 Plasmolyzability and gram-specificity, 120, 121
 Plasmolysis forms in bacteria, 122*
 Plasmolysis of bacteria, 120ff
 Plasmolysis of metaphyte cells, 59, 84
 Plasmolysis site in bacteria, 122*
 Plasmoptysis, 129
 Plate count procedure, sources of errors in, 131, 132
 Plate culture method (soil bacteriology), 131, 132
 Polar necrosis in bacteria, 129, 130*
 Potassium nitrate effect on bacteria, 124
 Primuline, 21, 30, 44
 Proteins, electrical charge of, 24, 25*, 26, 67-69
 Protoplasmatics, 120
 Protoplasm death, 46, 58, 60, 61, 64, 65, 69, 70
 Protoplasm, irreversible structural statics of, 71
 Protoplasm, necrobiotic state of, 71, 73, 80
 Protoplasm, reversible structural statics of, 69-70
 Protoplasm, state of, 68, 69
 Protoplasm, state of full vital activity, 69
 Protoplasm, state of limited vital activity, 70
 Protoplasm, structural dynamic state of, 69, 70; structural static state of, 70, 71
 Pus, 85, 88, 105, 106
 Pyronine, 19, 25, 26, 29, 33, 69; solubility in benzene, 19, 25, 26

 Quantity of acridine orange storage by yeast cells, 73, 74*, 76*, 77
 Quinine sulfate, 19
 Quininic, 22

Radiation injury to bacteria, 93, 100
 Rapid laboratory method of vital fluorochroming of bacteria, 85-88
 Reaction of bacterial protoplasm and metaphyte protoplasm to acridine orange, 101-104
 Red blood corpuscles, reaction to acridine orange fluorochroming, 87, 88
 Resistance of tubercle bacteria to acridine orange, 118*
 Resistance of yeast cells to acridine orange, 72, 77, 79, 80, 81*
 Resistance physiology of bacteria, 126ff
 Resistance to drying, bacterial, 127-129
 Resistance to drying in various drying media, bacterial, 128
 Resistance to drying of tubercle bacteria, 117, 118, 129
 Rhizosphere, 147
 Rhodamine B, 21, 30
 Rhoduline orange, 33
 Rivanol, 33, 48, 69, 105

Sarcina lutea, 26-31, 113
 Silver nitrate, 84
 Size of soil bacteria, 135, 137*
 Smallpox vaccine virus, 43
 Smallpox virus, 44
 Smear preparations, 16ff, 117
 Sodium 3-aminopyrene trisulfate, 46
 Sodium oxypyrene trisulfate, 5, 35, 38
 Soil algae, 139
 Soil bacteria, 131ff
 Soil fungi, 139
 Soil yeasts, 138
 Solvent for acridine orange in vital fluorochroming, 96, 97, 103, 104*, 105, 115
 Sources of error in counting bacteria in soil, 139
 Spermatozoa, 46, 66, 101
Spirillum, 30, 90, 100, 130
 Spontaneous movement of fluorochromed bacteria, 96, 99*, 100
 Spores, Hosema, vital fluorochroming of, 119
 Spores, Phycomyces, vital fluorochroming of, 82*, 83*
 Sporulation of myxomycetes, 46
 Sputum, 88, 119
Staphylococcus aureus, 30, 31, 88, 107-113
 Stokes' law, 4
Streptococcus, 94, 113
 Submicroscopic structure of protoplasm, 68-71
 Sulfonamides, 101, 106, 107
 Sulforhodamine G, 35
 Swelling of Phycomyces spores, 82*, 83*
 Swelling of protoplasm and fluorochroming, 62

Table salt effect on bacteria, 104, 115, 116, 122-124
Testing of filter combinations for fluorescence microscopy, 10
Testing of toxic effect of acridine orange on bacteria, 92-99, 102
Test tube method of fluorochroming of microorganisms, 79, 91
3,6-tetramethyldiaminoacridine, 48*
Thermoluminescence, 5
Thiazole yellow, 42, 43
Thymonucleic acid, 62, 102
Toluidine blue, 24*
Tonoplast, 73
Toxic effects of acridine orange on bacteria, 91, 104*
Trypanosomes, 43, 87; fluorescence-microscopic early identification of, 43, 87

Uranium glass, 14*
Urea effect on bacteria, 122, 124
UV-light fluorescence-microscopy, use of, 15, 16

Vacuole, 57, 61, 62, 64, 65, 66, 72, 73, 78, 82*, 83*, 86
Vacuoles, bacterial cell, 91, 99, 102
Vacuole contraction, 57, 59
Virulence of vitally fluorochromed bacteria, 93, 118
Virus particles, 43, 44
Vital fluorochroming of bacteria, 46ff, 99ff; of protoplasm, 45ff; of protoplasm with potassium fluorescein, 45; of vertebrates, 47
Vital fluorochroming with acridine orange, 44ff
Vital fluorochroming with tryptaflavin, 45
Vital staining, 44ff
Vital staining of bacteria, 46, 83ff, 99ff; of protoplasm, historical, 44, 45; of protoplasm, problems in, 44, 45
Volutin bodies, 73, 87

Water blue-acridine orange, consecutive method, 111-114; simultaneous method, 111
Water blue, fluorescence quenching with, 88, 110-114

Yeast, 46, 68, 69, 71ff, 74*-76*, 77ff, 81*, 90

Zooglea, 69
Zoogleas in the soil, 135-137*, 141-149
Zooglea value, 141-143

- END -

5214
CSO: R-7152-D